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**BLOOD RHEOLOGY, HAEMOSTATIC FUNCTION AND PERIPHERAL BLOOD  
FLOW IN DIABETES MELLITUS**

**A study of the Effects of Improved Blood Glucose Control and Treatment with  
Biosynthetic Human Insulin**

© Diana Margaret Ritchie

**Submitted in Fulfilment of the Requirement for the Degree of Doctor of Medicine**

**The University of Glasgow**

**The Work in this Thesis was conducted in the University Department of Medicine at  
Leeds General Infirmary**

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## SUMMARY

The aims of the studies were to examine blood rheology, haemostatic factors and peripheral blood flow in diabetes mellitus in relation to treatment and the presence of vascular damage. First, studies of the specificity of positive pressure filtration of dilute erythrocyte suspensions as a measure of erythrocyte deformability were made. Second, the effects of improved blood glucose control (achieved by outpatient management) on whole blood viscosity, haemostatic factors, peripheral blood flow and peripheral nerve function was examined. Third, a double-blind crossover study of the effects of treatment with biosynthetic human insulin (BHI) and purified porcine insulin (PPI) on blood rheology, haemostatic factors and peripheral blood flow was carried out.

The subjects studied included, i) newly diagnosed Type I diabetics ii) longstanding Type I and Type II diabetic patients with diabetic retinopathy and, iii) a normal control group. The effects of improved diabetic control were examined in both newly diagnosed Type I and established Type I and Type II diabetics. In the established Type I diabetics, this period formed the run-in to the BHI and PPI study. Each study insulin was administered for 90 days.

### 1) Erythrocyte Deformability

The number of residual leucocytes contaminating the erythrocyte suspensions was a major determinant of suspension filterability in diabetic patients,  $p < 0.0001$ . It was concluded that the filtration of dilute erythrocyte suspensions is not a specific measure of erythrocyte deformability. Diabetic control was not related to suspension filterability.

### 2) The Effects of Improving Diabetic Control

Improvement of diabetic control was confirmed by significant fall in  $HbA_{1C}\%$ .

#### i) Whole Blood Viscosity

Whole blood viscosity was largely determined by haematocrit. At comparable haematocrits, whole blood viscosity was approximately 9% higher in diabetic patients ( $p = 0.0029$ ). Improvement of diabetic control had no effect on viscosity measurements. Newly diagnosed patients were not examined.

## ii) Haemostatic Factors

### a) Fibrinogen

Levels were 41% higher in diabetic patients compared with normals ( $p=0.0005$ ). Independent predictors of high fibrinogen levels were poor diabetic control, high blood pressure, the presence of deteriorating retinopathy, and treatment with oral anti-diabetic agents. Improved diabetic control did not result in any change in fibrinogen.

### b) Factor VIII coagulant activity (VIII:C)

VIII:C was not elevated overall in diabetic subjects compared with normals; but concentrations of VIII:C were 18% lower in patients on oral anti-diabetic agents (Type II) compared with insulin-treated patients (Type I) ( $p=0.0198$ ). Type I patients had higher HbA<sub>1c</sub> levels than Type II patients ( $p=0.0033$ ). High HbA<sub>1c</sub>% was an independent predictor of high levels of VIII:C, but other factors included long duration of diabetes mellitus, the presence of deteriorating diabetic retinopathy and treatment with insulin. Improvement in diabetic control had no significant effect.

### c) Von Willebrand Antigen (vWF:AG)

VWF:AG levels were not elevated overall in diabetic patients compared with normal subjects, but levels were 36% lower in Type II patients compared with Type I patients ( $p=0.0016$ ). Independent predictors of vWF:AG were the same as those of VIII:C. Improved diabetic control resulted in a 13% fall of vWF:AG% ( $p=0.0065$ ).

### d) Ristocetin Co-factor

Again overall ristocetin co-factor was not significantly different from normal, but levels were 33% lower in Type II patients compared Type I patients ( $p=0.0005$ ). Levels were 32% elevated in Type I patients compared with normals ( $p=0.0398$ ). High FBG (but not HbA<sub>1c</sub>) was an independent predictor of high ristocetin co-factor%. Other independent predictors were high diastolic blood pressure and treatment with insulin. Improved diabetic control had no effect.

### e) Fibrinolytic Activity

Fibrinolytic activity was assessed by measuring ECLT. Diabetic patient ECLT's were not significantly different from normal. Independent predictors of lesser fibrinolytic activity were poor diabetic control (high HbA<sub>1c</sub>) and high diastolic blood pressure. Improving diabetic control had no effect on ECLT's.

#### f) Fibrinopeptide A (FPA)

These were measured in established Type I and the normal volunteers. FPA was greatly influenced by venesection technique. Levels were not significantly different in diabetics. In diabetics, an association of higher FPA with higher subject blood pressure was noted,  $p < 0.04$ .

#### iii) Peripheral Blood Flow

##### a) Calf and Forefoot

Resting calf (RCF) and forefoot (RFF) flow and reactive hyperaemia in the forefoot, following a 4 minute arterial occlusion, were measured by plethysmography. RCF and RFF were not significantly different from normal but vasodilation following ischaemic stress was 35% reduced in diabetes ( $p < 0.0001$ ). In newly diagnosed patients, an increase in peak flow (vasodilation) and a decrease in time to reach peak flow (TPF) was noted after commencing treatment. No effects of improving diabetic control were noted in established diabetic patients. In established Type I and Type II diabetics, nerve conduction times were studied, and an association of Common Peroneal "F" latency and TPF was noted.

##### b) Toe-pad

Toe-pad skin blood flow was measured using a Laser Doppler Flowmeter. There was a marked delay in TPF following arterial occlusion of the forefoot of diabetic patients, suggesting impairment of vasodilation of small vessels.

##### c) Peripheral Nerve Conduction Studies

Fourteen established diabetics who participated in the crossover study of BHI and PPI insulins were studied. Improved diabetic control resulted in shortening of "F" latency times and quickening of motor conduction times in the Ulnar and Common Peroneal nerves ( $p < 0.05$ ). There was no associated clinical improvement in nerve function.

#### 3) Double-Blind Study of Treatment with BHI and PPI

Diabetic control showed a tendency to deteriorate on treatment with BHI, although HbA<sub>1c</sub> levels were not significantly different. No effects on whole blood viscosity, haemostatic function factors, peripheral blood flow or peripheral nerve conduction times were noted.

These studies suggest that whilst many of the factors associated with diabetes mellitus are related to diabetic control, minor improvements in blood glucose as can be achieved by closer patient supervision do not result in substantial changes. Treatment with BHI

does not have any advantageous effects on blood rheology, haemostatic function or peripheral blood flow or peripheral nerve conduction. The relationship of the various factors studied to the development of diabetic vascular damage is discussed.

## ABBREVIATIONS

- art debt arterial debt, calculated by multiplying the resting flow value by the time of arterial occlusion.
- BHI Biosynthetic Human Insulin.
- $^{\circ}\text{C}$  Degrees celsius.
- cp Centipoise.
- C Chlorpropamide.
- CV Coefficient of Variation.
- ECLT Euglobulin clot lysis time.
- EDTA Ethylene diamine tetra acetic acid.
- F Female.
- FBG Fasting blood glucose.
- Fib Fibrinogen.
- FPA Fibrinopeptide A.
- G Glibenclamide.
- HbA<sub>1</sub>C Glycosylated haemoglobin.
- Hct Haematocrit.
- Hg Mercury.
- IDD Insulin dependent diabetes, Type I.
- MCV Motor (nerve) Conduction Velocity.
- N Number.
- M Male.
- mPa milliPascal.
- Me Metformin.
- NA Not Available.
- NIDD Non-Insulin Dependent diabetes, Type II.
- NR Not Recordable.
- NS Not Significant.
- PF Peak Flow (millilitres per 100 millilitres of tissue).
- PF-RF Peak post stimulus flow minus resting basal flow (millilitres per 100 mls of tissue).
- PI ECLT Euglobulin clot lysis time measured on a blood sample taken 1 hour after subcutaneous injection of insulin.
- PPI Purified porcine insulin.
- PPSF Peak post stimulus flow (millilitres per 100 millilitres of tissue).
- PPSF-RFF Peak post stimulus flow minus resting basal blood flow in the foot.
- PV ECLT Euglobulin clot lysis time measured on a sample taken after venous occlusion at 90 mmHg for 10 minutes.
- RBC Red Blood Cell.

- RCF Resting calf flow (millilitres per 100 millilitres of tissue).
- RFF Resting foot flow (millilitres per 100 millilitres of tissue).
- Rs Spearman's coefficient of variation.
- SCV Sensory (nerve) conduction velocity.
- SD Standard deviation of the mean..
- SE Standard error of the mean.
- TPF Time to reach peak flow values following arterial occlusion expressed in seconds.
- T<sub>50</sub> The half life of reactive hyperaemia following arterial occlusion expressed in seconds.
- VIII:C Factor VIII: coagulant activity.
- VWF:Ag Von Willebrand related antigen, occasionally in literature referred to as factor VIII antigen, VIII:AG, or factor VIII related antigen, VIIIIR:Ag.
- WCC White cell count.
- x Mean.
- (0-30%) The percentage of arterial debt (following arterial occlusion) paid back in the first 30 seconds of reactive hyperaemia.

## CHAPTER 1

## INTRODUCTION

**1-1 HISTORICAL PERSPECTIVES OF THE VASCULAR COMPLICATIONS OF DIABETES MELLITUS****1-1-1 General Background**

Diabetes mellitus is perhaps best considered as a heterogeneous group of disorders resulting in hyperglycaemia. Since the introduction of insulin treatment in the early 1920's, comparatively few people now die from the acute effects of uncontrolled hyperglycaemia but the complex metabolic effects and the long-term sequelae of the diabetic state remain a major cause of morbidity and reduced life expectancy in Britain and other industrialized societies. Indeed, the development of vascular complications is so characteristic of long-term diabetes mellitus that it is virtually regarded as part of the disease and the diagnostic criteria of both the American Diabetes Association (National Diabetes Data Group, 1979) and the World Health Organization (WHO Expert Committee on Diabetes, Second Report, 1980) are based on blood glucose levels which in epidemiological studies have been associated with the development of this specific diabetic tissue damage.

**1-1-2 Atherosclerosis and Diabetes Mellitus**

The long-term neurological, renal, eye and vascular complications were recognized and well described by the end of the nineteenth century but attracted little attention until after the introduction of insulin, when death from these causes became more common than death from acute metabolic disturbance. The excess of cardiovascular mortality was observed by \*Levine in 1922. In 1924, \*Labbe and Lefantin noted that arterial calcification was a frequent finding in diabetes mellitus, although it was 1968 before \*Neubauer reported on the distinctive features of this calcification which occurs particularly in the media of the arterial wall. Gangrene of the lower limb was found to be forty times more common in diabetic patients surviving from the 1920's onwards (\*Bell 1958) and diabetic patients with peripheral vascular disease were found to be more prone to the development of gangrene (\*Hines and Barker, 1940). This atheromatous disease of diabetes was found to involve more distal arteries than is found in non-diabetic individuals (\*Semple, 1953) with involvement extending to the metatarsal arteries (Ferrier, 1967). Diabetic atheromatous plaques were found to contain higher concentrations of mucopolysaccharides (\*Randerath and Dietzel, 1959) ash, calcium and cholesterol (\*Hevelke, 1955). Thus diabetic patients appear to be at risk of developing a more severe form of atheromatous disease than non-diabetics,

involving more distal arteries and with heavy calcification of the media and increased susceptibility to gangrene (\*as reviewed by Christensen, 1972).

### 1-1-3 Diabetic Microangiopathy

Appreciation that there was also a degeneration of vessels smaller than arteries, the microvessels, came later. Changes in the microvessels of diabetics were described by Woltman and Wilder in 1929 but comprehensive studies of the microvasculature in diabetes mellitus did not begin until the late 1950's. In diabetic microangiopathy two types of lesion tend to occur: i) accelerated thickening of the basement membrane with an assimilation of PAS positive material which in immunohistological studies corresponds to various plasma proteins (Dustin 1962) and ii) endothelial cell proliferation together with basement membrane thickening. The presence of increased vessel thickness and PAS stainability was described by Fagerberg in 1959, and the presence of increased basal membrane thickness by Aagenaes and Moe in 1961. Goldenberg and his colleagues drew attention to the enlargement of endothelial cells with cell proliferation in 1959. These vessel changes were noted first in intraneural vessels by Fagerberg and gave rise to the vascular hypothesis of diabetic neuropathy, which proposed that diabetic neuropathy was the result of impaired vascular supply to the affected nerves. However, descriptions of similar morphological changes in the capillaries of other tissues in diabetes proved the vessel damage to be a generalized problem whose functional importance depended on the response of the organ or tissue affected. In diabetes mellitus these microvessel lesions are most critical in the eye and kidney where they are responsible for the development of diabetic retinopathy and diabetic nephropathy. Death from cardiovascular diseases and diabetic nephropathy are the major causes of reduced life expectancy in diabetics of whom a considerable proportion will also suffer amputation of a limb and blindness before death. The prevention of these long term vascular complications is a major challenge for contemporary medicine

### 1-1-4 Current Perspectives

Clinical diabetic practice is now based on the generally accepted view, that the development of vascular complications is related in some way to the duration and degree of hyperglycaemia but there is little evidence that once complications have occurred that good diabetic control will reverse the situation (Tamborlane et al, 1982; Lauritzen et al, 1983; Viberti et al, 1983). In order to develop a rational treatment policy, a more thorough understanding of the changes that lead to the development of diabetic vascular disease and the relationship of these changes to diabetic control is necessary. Consideration needs also to be given to the cost, physiological, financial and emotional, of achieving good control against the benefits, which may vary between



diabetic patients depending on their predisposition to, or the presence of diabetic complications

Little has been achieved in the past sixty years since the introduction of insulin in the 1920's in terms of the prevention of diabetic complications. During the 1970's technical advances and the development of genetic engineering led to a second major development in the treatment of diabetes with the mass production of biosynthetic human insulin. The principal impact of biosynthetic human insulin appears to be that it represents a potentially limitless supply of insulin for the world's diabetic population. However, each new therapeutic option has to be examined for its specific therapeutic advantages. In human diabetes, human insulin might be considered a more physiological choice of treatment but clear therapeutic advantages have yet to be shown. The work presented in this thesis concerns itself chiefly with the control dependency or otherwise of various haemostatic, haemorreological and blood flow factors, all of which are thought to be involved in the development of diabetic microangiopathy and examines the therapeutic effects of treatment with biosynthetic human insulin on these variables.

## 1-2 THE MORTALITY AND MORBIDITY OF DIABETES MELLITUS.

Diabetes mellitus is essentially a biochemical diagnosis. Broadly speaking diabetic patients fall into one of two main groups, Type I) insulin dependent or, Type II) non-insulin dependent. In both groups there is a defect of glucose metabolism, in Type I due to absolute insulin deficiency and in Type II due to impairment of insulin action. Both insulin deficient Type I and hyperinsulinaemic Type II diabetics develop diabetic complications but in these two main groups the pattern of chronic tissue damage varies. Type I diabetes is associated with a mortality rate five times greater than the normal population. Before death one third of these patients will be registered blind in one eye largely due to retinopathy. Whilst some deaths are still the result of acute metabolic derangement, deaths from myocardial infarction and renal failure resulting from diabetic nephropathy are the most significant causes of death. When diagnosed before the age of 30 years the life expectancy for these patients is 29 years - only half will live to the age of 50 (Deckert et al 1978, [a]). Of the 10-15 percent of Type I diabetics who live more than 40 years, 10 percent will be blind, largely from retinopathy, another 10 percent will have proliferative retinopathy, but as a whole they are relatively free of serious morbidity. The mortality rates of non-insulin dependent Type II diabetes mellitus are harder to define as these patients may be asymptomatic for many years. Blindness in these patients is more often due to cataract and exudative retinopathy than proliferative retinopathy. Foot ulceration is commoner than in Type I diabetes but diabetic nephropathy is rarer. This different pattern of diabetic tissue damage may reflect the generally later age of onset of Type II diabetes and thus shorter duration of exposure to hyperglycaemia, or basic differences in susceptibility

and treatment. The prognosis of non-insulin dependent diabetes mellitus is worse for those patients who develop the disease at a young age (Oxford Textbook of Medicine 1983 [a])

### 1-3 THE RELATIONSHIP OF DIABETIC CONTROL TO THE DEVELOPMENT OF VASCULAR COMPLICATIONS.

#### 1-3-1 Atherosclerosis and Diabetes Mellitus

It is becoming clear that susceptibility to large vessel atherosclerosis and to diabetic microangiopathy are not synonymous. Whilst the incidence of microvascular complications appears to be fairly uniform in diabetic patients in different cultures and ethnic group exposed to equal hyperglycaemia, rates of atherosclerosis vary, (Jarrett and Keen, 1976) suggesting that the effects on the vasculature of hyperglycaemia may be influenced by other factors. In populations with a high incidence of atherosclerosis, the presence of even abnormal glucose tolerance as well as diabetes mellitus is associated with a sharp increase in the incidence of atherosclerosis, but there is less evidence of a relationship between duration and severity of hyperglycaemia and increased susceptibility to atherosclerotic disease of arteries. Susceptibility to peripheral vascular and ischaemic heart disease appears to be increased by the presence of hypertension, obesity, hyperlipaemia (particularly hypercholesterolaemia) and cigarette smoking. However, the excess of atherosclerotic disease in diabetic patients appears to be greater than that which would be predicted by these factors. Whilst it is generally held that the incidence of diabetic complications in a population is related to both degree and duration of exposure to hyperglycaemia, it is also accepted that the susceptibility of the individual to the effects of hyperglycaemia varies widely (West, 1982).

#### 1-3-2 The Early Studies of Diabetic Microangiopathy

From the 1950's onwards, many studies have attempted to define the relative importance of genetic predisposition, diabetic control and environmental factors to the development of diabetic complications, particularly to diabetic microangiopathy. The pathogenesis of diabetic microangiopathy is undoubtedly multifactorial. Genetic susceptibility, (Barbosa and Saner, 1984) the male sex (Bodansky et al, 1982), social habits including heavy alcohol consumption (Young et al, 1984) and cigarette smoking (Christiansen, 1978) and the presence of hypertension (Kornerup, 1957; Knowler et al, 1980; Hasslacker et al, 1985) have all been reported as risk factors but severity and duration of exposure to hyperglycaemia has been the only factor consistently linked with the development of microvascular damage. The first studies examining the relationship of diabetic control to the development of diabetic microangiopathy, reported in the 1950's and 1960's, were largely retrospective and the methods available

for assessing diabetic control were less accurate than those at present. However, even in some of these early studies (\*as reviewed by Tchobroutsky, 1978) many authors including \*Jackson et al (1950), \*Colwell, (1966) and \*Caird et al (1969), concluded that good control of blood glucose was beneficial in diabetic patients as regards the development of chronic complications. However there were others who found no such certain association between control of blood glucose and complications, \*Knowles (1964), \*Bondy and Felig (1971), \*Kaplan and Feinstein (1973) and \*Raskin (1978).

### 1-3-3 The Recent Studies of Diabetic Microangiopathy

Interpretation of these largely retrospective studies was complicated by many factors, not least the unknown influence of the tendency for diabetic control to improve once diabetic complications are discovered. In more recent years, the results of several large retrospective (Deckert et al, 1978 (b)) and prospective studies of diabetic control and the development of diabetic complications have become available (Job et al, 1977; Pirat, 1977; West et al, 1982; Howard-Williams et al, 1984, ). In these long-term studies hyperglycaemia has emerged as the major risk factor for the development of diabetic microangiopathy. Other evidence supporting the relationship of blood glucose and the occurrence of microangiopathy is derived from work with experimental animal models where the severity of diabetic complications is related to the severity of hyperglycaemia, (Rasch, 1979, [a, b, & c]) and it has been noted in man that lowering of blood glucose levels towards normal may result in reversal of many of the changes which accompany the diabetic state, such as slowing of nerve conduction times (Gegersen, 1968), hormonal disturbances, the presence of proteinuria, abnormally high glomerular filtration rates, abnormalities of lipid and amino acid metabolism (Tamborlane et al, 1979 [a & b]), all factors which may play a role in the development of complications. However, others argue that control of glucose levels is not the single cause of diabetic vascular damage, noting that even with poor control of glycaemia some patients remain well whilst others exposed to mild hyperglycaemia develop severe complications (as reviewed by West, 1982). Not all the recent studies of blood glucose control and the development of vascular complications show a positive relationship. The University Group Diabetes Program, a long-term prospective trial of treatment of asymptomatic adult onset diabetes (Type II) found that despite differences of glucose levels within different treatment groups, there were only minor differences in the occurrence of fatal or non-fatal events, (Knatterud et al, 1978). The results of this study have been criticized on the basis of the low incidence of diabetic retinopathy and often doubtful diagnosis of diabetes in the patients studied which may limit the scope of its conclusions.

In recent years, the relationship of blood glucose control to the development of complications has been more or less accepted and attention has focused on the therapeutic effects of improving diabetic control when microangiopathy has already

occurred and on elucidating the pathogenetic mechanisms of microangiopathy. Whilst long-term maintenance of good diabetic control remains the best therapeutic option for the prevention of diabetic complications, the effects of improving diabetic control in patients with pre-existing diabetic vascular damage are less obviously beneficial, and there is evidence of worsening of microangiopathy on institution of strict control of blood glucose levels (Hooymans et al, 1982). Whilst after a certain stage microvascular damage may be irreversible, the possibility of adverse effects of reversal of hyperglycaemia or of the specific regimes used to achieve near normoglycaemia has also to be considered. Near normal blood glucose levels can be obtained by treatment with continuous subcutaneous insulin given by infusion pump but patients are at increased risk of both hyperglycaemia if the pump fails and probably also hypoglycaemia (Pickup et al, 1985). In addition, these devices are expensive and thus not widely available and patients require extensive management and access to immediate medical advice in case of technical failure or medical problems. Considerable improvements in blood glucose control can also be achieved by more intensive management of diabetic patients with conventional multiple subcutaneous injection regimes but whether glucose levels are lowered sufficiently to achieve physiological benefit, without the same and with additional disadvantages to those of continuous insulin infusion devices has yet to be established.

Amongst the complex metabolic and hormonal changes resulting from the presence of hyperglycaemia, changes in the blood's rheological properties and the haemostatic system have been suggested as contributing directly to the development of vascular damage by impairing blood flow in the microcirculation and by predisposing to thrombosis. These changes in blood rheology and the haemostatic system and their influence on blood flow in diabetes mellitus are the main subject of this thesis and will now be considered in detail.

#### 1-4 WHOLE BLOOD VISCOSITY IN DIABETES MELLITUS

##### 1-4-1 Introduction

Rheology is the science of flow and deformation of matter. It is a term, which when applied to blood refers to its viscous properties and the deformability and interaction of its cellular elements, of which whole blood viscosity is an overall measure.

Skovborg and his colleagues reported in 1966 that whole blood viscosity was raised in diabetes mellitus and postulated that this increased viscosity might impair flow in the microcirculation, resulting in the intravascular erythrocyte aggregation and slowing of flow in small vessels in the retina reported earlier (as reviewed by Ditzel 1968).

Skovborg suggested that deoxygenation subsequent to these disturbances of blood flow could promote the structural changes of microangiopathy and theories, that increased blood viscosity in association with hypercoagulability, might contribute to thrombus

formation and a final occlusive event in the microvessels, are still accepted (in Joslin's Diabetes Mellitus, 1985). Whilst there have now been many reports of abnormal blood rheology, including raised blood viscosity, decreased erythrocyte deformability and increased erythrocyte aggregability in diabetes mellitus, evidence that these abnormal rheological parameters contribute to either the development of macrovascular or microvascular disease still rests purely on clinical correlation. At extremes of blood hyperviscosity due to either an increase in plasma viscosity, caused by excess of large proteins, as in Waldenström's macroglobulinaemia, or loss of deformability of the erythrocytes, as in sickle cell disease, blood flow in the microcirculation is impaired and examination of retinal blood vessels reveals vessel dilation, increased tortuosity, clumping of erythrocytes within the vessel leading to segmentation of dilated vessels and retinal haemorrhage (see the Oxford Textbook of Medicine 1983 [b]). Gross impairment of flow in the microcirculation also occurs in polycythaemia vera, where the cell volume is greatly increased but the effects of a much more modest elevation of blood viscosity, as reported in diabetes mellitus, are less obvious.

#### 1-4-2 Theoretical Aspects of Whole Blood Viscosity and Blood Flow

The French physician Poiseuille is widely regarded as being the first to examine the dynamics of flow in capillary sized tubes. However, it was \*Girad (1813-15) who defined the relationship of flow, pressure and tube length and \*Hagen (1839) who noted that flow in fact varied with the 4th power of the radius. \*Poiseuille (1840) modified the formulae by K, a constant relating to the viscosity of the fluid. Although correct in principle, Poiseuille's equation was refined mathematically by \*Wiedemann (1856) and \*Hagenbach (1860), but remains known as the Hagen-Poiseuille formula:

$$\text{Rate of Flow} = \pi/8 \times \Delta P \times r^4/L \times N,$$

where  $\Delta P$  = the pressure gradient,  $r$  = the radius of the vessel,  $L$  = the length of the vessel and  $N$  = viscosity. (\*see Skalak and Chien 1981 and Usami 1982).

Thus viscosity is a major determinant of flow although it must immediately be stated that the experimental data on which this formula is based were obtained using water, alcohol and mercury and that the rigid capillary tubes were only comparable in size but not in other characteristics to the dynamic tapering endothelial lined vasculature. The Hagen-Poiseuille formulae describes steady laminar flow in a Newtonian fluid in a straight tube without turbulence. In the human body blood flow is pulsatile, the architecture of the blood vessels is complex, the vessel wall is distensible and blood is a non-Newtonian fluid whose viscosity varies inversely with flow rate. However it is still true that the relation between pressure gradient and blood flow is a function of vessel geometry and blood viscosity and these latter two factors are in fact related.

### 1-4-3 Studies of Whole Blood Viscosity in Diabetes Mellitus

Skovborg's original observation of increased blood viscosity in diabetes mellitus has been confirmed by many others including (Hoare et al, 1976, Barnes et al, 1977, Lowe et al, 1980, Paisey et al, 1980; Poon et al, 1982; Caimi, 1983). The increase in whole blood viscosity found in diabetes, although described at high shear rates, is more obvious when measurements are made at low shear rates (as reviewed by Caimi, 1983). Increased whole blood viscosity has been found to be more marked in diabetic patients with vascular complications (Isogai et al, 1976; Barnes et al, 1977; Lowe et al, 1980) and in patients with poor metabolic control (Skovborg & Nielsen, 1969; Schmid-Schonbein and Volger, 1976; Paisey et al, 1980), conditions associated with increases in plasma proteins such as fibrinogen. As serum viscosity is not increased in diabetes mellitus (Mosora et al 1972), the increase in whole blood viscosity in diabetes mellitus would appear to be due to changes in plasma proteins and their interaction with erythrocytes. Although the presence of increased viscosity in diabetes is supported by many reports its relevance to the development of diabetic complications is not yet established.

### 1-4-4 The Influence of Haematocrit and Fibrinogen Levels on Whole Blood Viscosity in relation to Diabetes Mellitus

Blood viscosity when transformed logarithmically, is linearly related to packed cell volume (Begg and Hearn, 1966). The haematocrit in diabetes mellitus is often increased during periods of poor control because of dehydration. Even when corrections are made for elevation of packed cell volume, viscosity is still relatively elevated in diabetes mellitus and this appears to correlate with the presence of hyperfibrinogenaemia (Skovborg et al, 1966; Hoare et al, 1976; Barnes et al, 1977; Lowe et al, 1980; Poon et al, 1982). In studies of the effects of improved diabetic control on blood viscosity, an initial fall in viscosity occurs with rehydration and fall in haematocrit but the corrected viscosity remains elevated for some months until fibrinogen levels return to normal (Barnes, 1981). Thus the factors responsible for the increase in in vitro blood viscosity in diabetes mellitus appear to be increased haematocrit and raised levels of fibrinogen. Fibrinogen is an acute phase reactant protein and is elevated in a variety of disorders besides uncontrolled diabetes mellitus and the presence of vascular diabetic complications. Addition of fibrinogen has been shown to increase blood viscosity in vitro, but within the range of fibrinogen normally found in man and at the flow rates normally encountered, the effects on viscosity are theoretically likely to be small, (Weaver et al, 1969, Rampling and Challoner, 1983). It has been questioned whether the elevation of whole blood viscosity in diabetes mellitus due to hyperfibrinogenaemia, calculated as being equivalent in one study to a change in haematocrit of 1.7% (normal range of haematocrit 14%) is of any significance (Poon et al, 1982). Mild hyperfibrinogenaemia and hyperviscosity are not unique to diabetes

mellitus and are found in a variety of conditions, not associated with the development of microangiopathy, including atherosclerosis, (Mayer, 1964, Dintenfass et al, 1966, Dormandy, 1970, Fuch et al, 1984), non-embolic cerebro-vascular disease (Marshall, 1982 ), oral contraceptive use (Oski & Lubin 1972), cigarette smoking (Dintenfass, 1975), alcohol intake (Galea and Davidson, 1983), and mild hypertension (Letcher et al, 1981). In polycythaemia vera, the incidence of vascular occlusive episodes is increased even at modest elevations of blood haematocrit and correlates positively with the haematocrit (reviewed by Stuart and Kenny, 1980). Whilst elevated blood viscosity is commonly found in diabetic patients in whom atherosclerosis is the major cause of death, more work is required before high haematocrit is established as a risk factor for atherosclerosis in these patients.

The increase in blood viscosity in diabetes mellitus is of a much lesser magnitude of that seen in the hyperviscosity syndromes but it is marked at low shear rates at which erythrocyte aggregability and deformability contribute to the non-Newtonian behaviour of blood. Specific defects of erythrocyte deformability have also been reported in association with diabetes mellitus and it has been suggested that these effects may also impede flow. The various methods which have been applied to the study of erythrocyte deformability will now be discussed.

## 1-5 ERYTHROCYTE DEFORMABILITY IN DIABETES MELLITUS

### 1-5-1 Definition of Erythrocyte Deformability

The deformability of erythrocytes can be defined as the ability of these cells to change shape when subjected to fluid shear. The normal erythrocyte is an extremely deformable cell and it is this property which allows erythrocytes to enter the smaller nutritive capillaries which are only about half their diameter. In theory, impairment of erythrocyte deformability might result in failure of erythrocyte perfusion of the small capillaries leading to poor tissue nutrition and it has also been suggested that the higher pressures exerted on the vessel wall during passage of these hardened erythrocytes might contribute to microvascular degeneration of diabetes mellitus.

### 1-5-2 Studies of Erythrocyte Deformability by Filtration Methods in Diabetes Mellitus

Reduced erythrocyte deformability in association with diabetes mellitus was first reported by Schmid-Schonbein and Volger in 1976. They measured deformability by examining the filterability of 10 percent erythrocyte suspension in autologous cell-free plasma comparing 45 control subjects with 77 'early and mature' diabetic subjects. The diabetic group had different duration of disease, variable metabolic status which was assessed by 24 hour urine glucose excretion and post-prandial blood glucose levels and various degrees of complications which were defined as either no retinopathy, background retinopathy or proliferative retinopathy by direct fundoscopy. Filterability

was examined by determining the flow rate of the erythrocyte suspensions through 5 micron Nucleopore sieves under a driving pressure of 5 cm of water compared with that of autologous plasma. The difference in flow rates of plasma and of erythrocytes suspended in plasma through the capillary sized channels of the Nucleopore filter was assumed to be a measure of the ability of the erythrocytes to deform, a pre-requisite for passage through the filter pores. Erythrocyte filterability was found to be reduced in all the diabetics, showed no correlation with duration of disease or presence of diabetic complications but did appear to correlate with the current metabolic control, in that of two groups of diabetic patients, one with high and one with low 24 hour glucose excretion and glucose levels, filterability was lower in the high 24 hour glucose excretion group and most of all in 6 patients who presented with diabetic coma. In vitro studies of the effects of lactate, acetoacetic and free fatty acid ketone bodies and lowering of pH were all found to harden erythrocytes from both normal and diabetic subjects. Further studies of erythrocyte filterability in diabetes mellitus were in essential agreement with Schmid-Schonbein and Volger's original findings. Reduced whole blood filtration in diabetes mellitus was also reported by Barnes and his colleagues (1977) who examined whole blood filterability, under the effects of a negative pressure of 20 centimetres of water, a method introduced by Reid et al (1976[a]). Thirty-six diabetic subjects were examined and the lowest filterability was found in those diabetic patients with extensive microangiopathy. In their first study, Juhan and colleagues (1978) examined whole blood filtration, using a similar method but employing 5 micron millipore filters (which are now regarded as being unsatisfactory because of the non-uniformity of the pores). Forty diabetic in-patients, twelve arteriopathies from a vascular ward and fourteen control subjects were examined. Again the diabetic group were heterogeneous in terms of diabetic control, treatment and presence of diabetic complications, although details of individual patients were not supplied. Whole blood filterability was found to be reduced in the diabetic patients, particularly in diabetic patients with atheromatous disease and also in the non-diabetic group with vascular disease. There was no apparent difference of filterability in the insulin dependent and non-insulin dependent diabetics and no apparent difference in poorly and well controlled diabetics, although control was rather arbitrarily divided into two groups of 'controlled' and 'non-controlled' diabetes on the basis of fasting glucose levels. However, a tendency of increased filterability in the well-controlled insulin treated patients was noted. In subsequent studies, using essentially the same methods but employing the more reliable nucleopore filters and examining both whole blood and concentrated washed erythrocyte preparations Juhan et al (1981) and Drouin et al (1981) found that the abnormal deformability in insulin dependent diabetics was corrected by reversal of hyperglycaemia in vivo after treatment with insulin given using an artificial pancreas to control the blood glucose levels. Infusion of insulin also reduced the abnormality of erythrocyte filtration even when the hyperglycaemia was maintained by a feed back controlled infusion of glucose (Juhan et al 1981) and a



further report by the same group suggested that the defect in erythrocyte deformability could also be corrected in vitro by incubation with insulin (Juhan et al 1982). Considerable interest was expressed in these findings and various different factors such as glycosylation of haemoglobin and alterations in other factors associated with impairment of erythrocyte deformability in vitro such as alterations of the membrane lipid composition (Cooper 1978), were suggested as being responsible for this apparent diminution of erythrocyte deformability in diabetes mellitus. However, later research into the techniques of erythrocyte filtration cast doubt on the validity of these filtration tests as a specific measure of erythrocyte deformability. It has been recognized that plasma factors such as hyperprotinaemia resulting in erythrocyte aggregation and the haematocrit of the sample, elevated leucocyte counts and platelet activation also exert an influence on erythrocyte filterability (as reviewed by Boisseau, 1981). Thus in Schmid-Schonbein and Volger's study using autologous plasma as the suspending medium, filterability may have been influenced by fibrinogen levels producing erythrocyte aggregation. In Barnes's and Juhan's early studies on whole blood, the sample haematocrit, plasma factors, leucocytes and platelets may well have influenced the sample filterability. Whilst whole blood filterability appears to be a more physiological test, the overall opinion of workers in the field is now that filtration tests ought to be carried out on suspensions of washed erythrocytes in order to avoid these artifactitious effects.

### 1-5-3 Studies of Erythrocyte Deformability by the Micropipeting of Erythrocytes in Diabetes Mellitus

A variety of other methods have been used to measure erythrocyte deformability in diabetes mellitus including the micropipetting of individual cells. Micropipetting of erythrocytes as a measure of membrane viscoelasticity was introduced by Rand and Burton (1964) who modified the technique used by Mitchison and Swann for the study of marine egg stiffness. Problems with the micropipette technique are that it is time-consuming and cumbersome. Micropipettes of uniform bore of around 3 micron bore have to be produced and only small numbers of cells can be examined at any one time. For comparison studies the same delicate glass micropipette has to be preserved and used. Rand and Burton (1964) examined the pressure required to partially aspirate erythrocytes into the bore of the micropipette. McMillan et al (1978) examined the flow properties of individual erythrocytes in glass pipettes of 4 micron diameter by measuring the pressure gradient required to establish a standard oscillatory movement over a 130 micron path by an erythrocyte wholly aspirated into the micropipette, and also by recording the time taken for cells ejected from the pipette to recover their normal discoid shape. Paired studies of control and experimental erythrocytes were performed using the same pipette and examining seven cells from each sample. McMillan et al, (1978) found that the pressures required to oscillate diabetic

erythrocytes within the micropipettes was elevated by approximately 50 percent in both a heterogeneous group of 9 diabetic patients and in diabetic rats as compared with matched controls. Erythrocytes from subjects with hereditary spherocytosis, in which erythrocyte deformability is reduced, offered less flow resistance than diabetic cells. The diabetic cells were also slower to restore their shape on discharge from the micropipette. These findings were strongly suggestive of an increase in either the membrane or intra erythrocyte viscosity and McMillan et al, (1978) postulated that the presence of increased amounts of haemoglobin A<sub>1</sub>C might increase intra erythrocyte viscosity. However, when McMillan and Gion (1981) examined the viscosity of normal and glycosylated haemoglobins in solution they found these to be identical, which suggested that the chemical changes in haemoglobin in diabetes did not contribute to reduced erythrocyte deformability.

#### 1-5-4 Other Methods used to Assess Erythrocyte Deformability in Diabetes Mellitus

The micropipette technique is not suitable for the study of large numbers of different samples. In an attempt to find a suitable method for the study of large patient numbers, McMillan and his co-workers then looked at the rate at which erythrocytes in dilute suspension form doublets after settling to the surface of a microscope slide (McMillan et al, 1981). The rate of doublet formation was reduced in 20 diabetic patients when compared with 20 normal subjects but there was no association of doublet formation with fasting blood glucose, type of diabetes or presence of complications. The formation of cell doublet consists of elevation and subsequent apposition of one cell over another, a process limited by the ability of each cell to deform and by intercellular attraction. Although this is a simple rapid technique it is not yet established how doublet formation compares with other tests of erythrocyte deformability.

Yet another approach in evaluating erythrocyte deformability in diabetes has been the examination of shear induced deformation of cells, either by photographing the shape taken up by erythrocytes in a shear field (induced in a highly viscous suspension fluid) or by assessing the elongation ability of the erythrocytes by analysis of diffraction patterns from a laser beam (Kiesvetter et al, 1981 ). In both cases, an important determinant of deformability is the viscosity of the suspending media. Williamson et al (1981) first reported that shear induced deformation of erythrocytes was decreased in diabetes. The numbers in this study were small, only 5 control and 6 diabetic subjects and in a later study of 10 diabetic and 10 control subjects, this same group reported no significant differences between diabetic and normal subjects (Williamson et al, 1985).

Direct studies of erythrocyte membrane fluidity in diabetes, are also contradictory. Using an electron spin resonance method with stearic acid probes, Kamada and Otsuji (1983) found membrane fluidity to be reduced in diabetes mellitus. They found no

relation of impaired fluidity to diabetic control, blood glucose or duration of diabetes mellitus, but changes in erythrocyte membrane lipid classes were noted. The effect of insulin on human erythrocyte membrane fluidity was examined by Bryszewska and Leyko (1983) who found that addition of  $10^{-9}$  mol/l of porcine insulin significantly increased the membrane fluidity as measured by an optical method using the fluorescent label pyrene. No comment was made on differences in membrane fluidity between normal and diabetic erythrocytes as the small numbers, 13 diabetic patients and 6 normal control subjects probably precludes meaningful statistical analysis. In contrast to this study, Luly and colleagues (1981) reported that insulin at a similar concentration of  $10^{-9}$  M, decreased membrane fluidity as measured by fluorescence labelling with 1,6 diphenyl 1,3,5- hexatriene. They suggested that the reaction of insulin with its receptor altered the erythrocyte membrane microenvironment decreasing  $(Na^+ - K^+) - Atp$ -ase and membrane fluidity.

Thus, rheological studies in diabetes mellitus have produced confusing results, largely because of methodological problems, with little agreement over the presence of specific rheological abnormalities and no hard evidence of the physiological significance of these putative defects in vivo.

## **1-6 HAEMOSTATIC FUNCTION IN DIABETES MELLITUS**

### **1-6-1 Introduction**

Interest in the role of abnormalities of the haemostatic system in the development of both micro- and macro- vascular complications of diabetes mellitus has waxed and waned over the past 20 years. Haemostasis has been defined as "the spontaneous arrest of bleeding from a ruptured vessel" (MacFarlane, 1976) and is normally accomplished by a combination of three processes, i.e. the contraction of the vessels, the adhesion and aggregation of formed blood elements such as platelets and the process of blood coagulation. In man these vascular, cellular and biochemical haemostatic functions have evolved to a high degree of complexity and all are required for efficient haemostasis. Excessive clot formation may constitute as much a threat as catastrophic haemorrhage and is prevented by various homeostatic control mechanisms. Once the function of the haemostatic barrier has been served, fibrin is removed by the fibrinolytic enzyme system and the leucocytes, a process which leads to recanalization of damaged vessels. In addition it would appear that the haemostatic system is also involved in various other physiological and pathological processes including the inflammatory process through the release of kinins and activation of the complement system.

### 1-6-2 Evidence that an Excess of Haemostatic Factors is Thrombogenic

Most studies of the haemostatic mechanism have highlighted the effects of deficiency of any one of its components. From these studies it is obvious that deficiency of any one factor is generally still compatible with survival and that the various components are normally available in excess to that required for normal function. Little is known about the influence of relatively higher than normal circulating levels of haemostatic factors.

On the basis of evidence from various epidemiological studies in the general population, it has been suggested that high levels of blood haemostatic factors may predispose to the development of a hypercoagulable state which in turn predisposes to vascular damage. Positive associations have also been reported between fibrinogen levels and risk of stroke (Wilhelmsen et al, 1984), but the commonly quoted study is the Whitehall survey, a long-term prospective epidemiological study of 1510 male civil servants, where higher levels of factor VIII, factor VII and fibrinogen were found in individuals at who later died from cardiovascular disease as compared with survivors recruited at the same time (Meade et al, 1980). In a later report of the same study (Meade et al, 1986), factor VII and fibrinogen levels were found to be particularly high in those subjects who went on to develop myocardial infarction within the next 5 years. These results have been cited as evidence that thrombogenesis may play an important role in the development of coronary artery disease. However, although the mean follow-up period in Meade et al's study was 10 years at the time of the 1986 report, the development of coronary artery disease may take place over a longer period. It is possible that the high levels of various haemostatic factors simply mark out those individuals with pre-existing asymptomatic vascular damage.

As mentioned previously, the haemostatic system is also involved with the inflammatory process. A "haematological stress syndrome", (Annotation, 1979) is reported in association with surgery (Egeberg, 1962, Aronsen et al, 1972), trauma, acute infection, myocardial infarction and atherosclerosis (see Stuart et al 1981). The so called haematological stress syndrome consists of raised levels of blood platelets, leucocytes and various blood proteins including fibrinogen, antithrombin III, factor VIII and serum globulins, the latter hyperprotinaemia probably accounting for the hyperviscosity associated with atherosclerosis. Whilst, in surgery, trauma, acute infection and myocardial infarction, there is a predisposition to thrombosis, the thrombotic complications noted are chiefly those of venous thrombosis, pulmonary embolism and disseminated intravascular coagulation, conditions somewhat different from diabetic microangiopathy. However the different pattern of vascular problems may reflect on the interaction of disturbances of haemostatic function with other risk factors which determine the site of damage.

Circumstantial evidence has also linked hypofibrinolysis with atherosclerosis, venous thrombotic disorders and other diverse conditions such as cutaneous vasculitis, Bechet's disease, preclampsia, hyaline membrane disease (as reviewed by Davidson & Walker, 1981). The fibrinolytic system is the major component of the fibrin clearing mechanism and evidence that hypofibrinolysis might be atherogenic was provided by Kadish et al (1979) who found that endothelial cells became disorganized when contacted by fibrin. Fibrinolytic activity in the veins of the arms has been found to be much greater than that of the leg (Pandolfi et al, 1968) and it is the relatively hypofibrinolytic leg which is predominantly affected by atheromatous and diabetic microvascular damage. However studies of large vein fibrinolytic activity may not be relevant to the arterial circulation and small vessels.

Some evidence for a thrombogenic role of haemostatic factors is provided by animal studies. It has been noted that pigs with von Willebrand's disease are resistant to the development of atheroma and it has been postulated, that by promoting adherence of platelets to injured endothelial surfaces, von Willebrand factor might contribute to smooth muscle proliferation and the development of atheroma (as reviewed by Zimmerman & Meyer, 1981).

#### 1-6-3 Studies of Haemostatic Factors in Diabetic Patients

Following the first reports in the 1960's, which suggested that blood clotting times were shorter in diabetic patients (Egeberg, 1963), the coagulation system has been extensively studied in diabetes mellitus. Large numbers of studies have examined platelet function in diabetes. Several abnormalities of diabetic platelets are reported including hyperaggregability, (\*Kwaan et al, 1972), and reduced in vivo survival in diabetes mellitus, (Abramhansen, 1968; Ferguson et al, 1975; Paton 1979). These abnormalities have been associated with the presence of hyperglycaemia (Gonzalez et al, 1980; Peterson et al, 1977), microvascular complications, (Szirtes, 1970; Mayne et al, 1970; Heath et al, 1971), hyperlipidaemia (Betteridge et al, 1981) and the presence of higher levels of Factor VIII or Willebrand factor (Bensoussan et al, 1975).

Abnormalities of the prostaglandin pathway, with proportionally less production of the antiaggregatory prostacyclin and increased generation of the platelet aggregatory of thromboxane in diabetes have also been reported in diabetes mellitus, (Dollery et al, 1979 and Halushka et al, 1981). Insulin infusion has been reported as reversing platelet sensitivity to aggregation with ADP (Juhan et al, 1982 and Giugliano et al, 1982) but also conversely has increasing platelet hyperaggregability, (Jackson et al, 1984).

#### 1-6-4 Fibrinogen in Diabetes Mellitus

Studies of the coagulation factors in diabetes mellitus generally agree on the presence of hyperfibrinogenaemia, noted by Mayne et al, (1970). Whilst it is generally agreed

that the highest levels of fibrinogen are to be found in those diabetic patients who have vascular disease (Little and Sacks, 1977; Cederholm- Williams et al, 1981; Fuller, 1981), positive correlation of fibrinogen level with diabetic control as measured by HbA<sub>1</sub>C has also been reported (Coller et al, 1978) and treatment with insulin has been associated with higher levels than treatment with oral agents (Brooks et al, 1983).

#### 1-6-5 Factor VIII in Diabetes Mellitus

Factor VIII has been the most extensively studied of the coagulation factors with many reports of raised levels of the coagulant and of both von Willebrand components, antigenic and ristocetin cofactor associated activity. As mentioned, studies of the factor VIII complex have been prolific in the field of atherosclerosis but interpretation of the results of factor VIII studies in vascular disease remains vexed. In diabetes mellitus studies of the factor VIII complex give conflicting results. Elevated factor VIIC levels in diabetes mellitus are reported by Borkenstein & Muntean, 1982, Gensini et al, 1979, Bern et al, 1980, Vergani et al, 1981 and by Dornan et al, 1983). Raised levels of factor VIIC have been reported in diabetic children apparently free of vascular disease (Borkenstein & Muntean, 1982) and in this group of 86 insulin-dependent diabetic children factor VIIC was highest in a subdivision of these children who were designated as having poorly controlled diabetes mellitus on the basis of HbA<sub>1</sub>C levels and urinary glucose excretion. Duration of diabetes mellitus was not found to be of any significance as regards factor VIIC levels. Further suggestions that factor VIIC levels are related to diabetic control was suggested by Hughes et al, (1983), who found that treatment of hyperglycaemia resulted in lowering of factor VIIC levels in 36% of the group studied. Others have suggested that the highest VIIC levels are found in diabetics with vascular complications (Dornan et al, 1983) and this has led to suggestions that high levels of VIIC are related to the presence of vasculopathy. Other studies have been negative (Fuller et al, 1979; Cederholm-Williams et al, 1981 and Masperi et al, 1980). These discrepancies may in part be explained by the very wide range of normal factor VIIC levels. Christe et al, (1984), found that the factor VIII complex as well as other coagulation parameters including fibrinogen levels and euglobulin clot lysis times showed a relationship with age in both normal subjects and diabetic patients without vascular disease. In the cases of diabetic patients with vascular disease the relationship of factor VIIC and age and that of fibrinogen with age were obscure. Duration of diabetes does not appear to be an important factor but many of the variables discussed here i.e. age, duration of diabetes mellitus, increased incidence of vascular disease and poor diabetic control are related and without appropriate analysis an incomplete understanding will follow.

Studies of factor VIII von Willebrand factor in diabetes mellitus give similar mixed results. Cultured human umbilical endothelial cells respond to incubation with elevated glucose concentrations by increasing factor VIIR:Ag levels, (Mordes et al, 1983) but

there has been little other evidence to suggest that endothelial synthesis and/or release of factor VIII:Ag is related to hyperglycaemia rather than physical injury of the endothelium. Factor VIII:Ag (von Willebrand antigen) was also elevated in the diabetic children of Borkenstein & Muntean (1982), and raised levels are also reported by Gensini et al, (1979) and Dornan et al, (1983). Raised levels of factor VIII ristocetin cofactor activity were also reported by Gensini et al, (1979) and Lufkin et al, (1979). Lufkin et al, (1979) found that ristocetin cofactor was higher in diabetic patients with vasculopathy whereas there was a smaller increase in factor VIII:Ag levels. Both factor VIII ristocetin cofactor and factor VIII:Ag are secreted in increased amounts in diabetics compared with normals after venous occlusion, suggesting that larger amounts of these proteins may be stored by the diabetic endothelium (Giustolisi et al, 1980). Diabetics with proliferative retinopathy are reported to have particularly high levels of factor VIII von Willebrand factor compared to those with lesser degrees of retinopathy (Coller et al, 1978). Similarly Christe et al, (1984), found diabetic patients with vasculopathy had both higher levels of factor VIII:C, VIII:Ag (von Willebrand antigen) and particularly ristocetin cofactor, although VIII:Ag levels were also high in non-diabetic patients with vascular disease. Whilst elevation of factor VIII:C appeared to be specific to the presence of diabetes mellitus rather than vascular disease, ristocetin cofactor levels in the diabetic patients as a whole were positively correlated with diabetic control as measured by HbA<sub>1c</sub> levels. Patients with poor diabetic control, however, also tended to be the ones with vascular complications. Insulin-induced hypoglycaemia in both juvenile diabetics and normal subjects results in increased levels of factor VIII related antigen (Dalsgaard-Nielsen et al 1982) and factor VIII coagulant activity (Corrall et al 1980) though it is possible that the effects here are also mediated by concomitant hormonal changes such as adrenalin.

So far the evidence of a role of the factor VIII complex in the development of vascular disease lies solely on the basis of clinical correlations. However, it is noted that pigs with von Willebrand's disease are resistant to the development of atheroma (Bowie et al, 1975) less information is available on the development of atheroma or diabetic complications in humans with von Willebrand's disease.

#### 1-6-6 Other Fluid Phase Coagulation Factors in Diabetes Mellitus

Other coagulation factors have been less extensively studied in diabetes mellitus but there are also reports of raised levels of factor V and factor VII (Fuller et al, 1979). Whilst there are some negative studies of coagulation factors in diabetes, reports of factors inhibiting the coagulation process are more conflicting. Antithrombin III levels are variously reported as being normal (Gandolfo et al, 1980), reduced (Banerjee et al, 1974) and increased in diabetes (Grigiani et al, 1981). These discrepancies might be partly explained by different means of assessing antithrombin III levels as its biological

activity and immunologic activity do not appear to be one and the same thing (Gandolfo et al, 1980).

#### 1-6-7 Fibrinolytic Activity in Diabetes Mellitus

Studies of fibrinolytic capacity in diabetes mellitus are also hampered by methodological differences and inhomogeneity in the patients studied. Fibrinolytic activity can be measured directly by measuring the plasminogen activator activity of the vein wall (Pandolfi et al, 1968) or by measuring the fibrinolytic capacity of blood. In studies of blood, the presence of inhibitors of fibrinolysis which are not removed in some assays, must be taken into account (Kluft, 1979). Stimulation studies give a measure of the amount of plasminogen activator released from the vein on dynamic testing. The commonest of these is the venous occlusion test (Clarke et al, 1960, Robertson et al, 1972). Fibrinolytic capacity in diabetes mellitus is reported as being reduced (Fearnley et al, 1963; Almer and Nilsson, 1975), normal (Mackay and Hume, 1964; Tanser, 1967) and increased (Bellet et al, 1961). Obesity and old age, common to many diabetics, are associated with reduced fibrinolytic capacity (Fearnley et al, 1963, Hamilton et al, 1974) and both hypo (Dalsgaard-Nielsen et al, 1982) as well as hyperglycaemia have been reported to result in shortening euglobulin clot lysis times. Interesting studies of plasminogen activator content of vein wall in diabetes have reported that diabetic patients with retinopathy have normal plasminogen activator content of the vessel wall but on stimulation by venous occlusion, low blood levels of fibrinolytic activity. The converse was found in diabetics free of retinopathy, who had low levels of vessel plasminogen activator but high levels of blood fibrinolytic activity on stimulation testing (Almer and Pandolfi, 1976; Haitas et al, [a] 1984). These authors have speculated that the changes in patients with microvascular damage, might be due to faulty release of plasminogen activator from the vessel wall, which has predisposed them to vascular damage.

In addition to metabolic factors, fibrinolytic activity is also influenced by venous pressure. Fibrinolytic capacity is four times higher in the arm veins than in leg veins (Pandolfi et al 1968). Prolonged recumbency results in a rise in leg vein fibrinolytic capacity until it reaches values obtained in arm veins. It has been suggested, that the higher venous hydrostatic pressure associated with the upright position results in continuous plasminogen activator release and subsequent exhaustion of the leg vein wall content. Reduction of hydrostatic pressures allows restoration of the plasminogen activator content to arm vein levels (Karaca and Nilsson, 1971; Keber, 1983). Release of plasminogen activator in response to venous occlusion forms the basis of the commonly used venous occlusion test used for assessment of fibrinolytic potential (Robertson et al 1972). Prolonged venous occlusion results in exhaustion of plasminogen activator content which takes some 10 to 14 days to return to normal levels (Keber and Stegnar, 1982). Diabetic patients are commonly hypertensive (Drury,



1983) and vasomotor regulation is thought to be disturbed (Tooke, 1986). Whether these factors influence plasminogen activator release in addition to the many possible metabolic factors remains to be established.

#### 1-6-8- Evidence of a Thrombogenic Tendency in Diabetes Mellitus

Thus in diabetes mellitus, investigations of haemostatic function have suggested that there are abnormalities of several aspects of the haemostatic process. Diabetic platelets are hyperaggregable, (\*Kwam et al, 1972; Colwell et al, 1976 and many others), raised levels of various coagulation factors occur, (Mayne et al, 1970; Pandolfi et al, 1974; Fuller et al, 1979 and many others), abnormalities of the fibrinolytic mechanism are reported (Fearnley et al, 1963; Cash & McGill, 1969; Almer et al, 1975 and others) and decreased levels of regulatory proteins such as antithrombin III are also reported, (Banerjee et al, 1974; Ceriello et al, 1983). However the heterogeneity of diabetic patients combined with a paucity of intra laboratory standardization of haemostatic function tests account for many conflicting reports in the literature, particularly the studies of fibrinolytic activity and of concentrations of coagulation inhibitors. Correlations of altered haemostatic factors and the presence of diabetes mellitus are not proof of a hypercoagulable state but some evidence of enhanced thrombogenesis might be provided by studies of fibrinogen turnover. If the coagulation process is activated, the turnover of fibrinogen - the end product should be increased and that there should be evidence of fibrin deposition. Fergusson et al, (1975) reported that mean fibrinogen survival in 11 diabetic subjects was 4 days compared with 6.9 days in 7 normal volunteers. These results were substantiated by reports from Banerjee et al, (1973) and by Jones and Peterson (1979) who found reduction of fibrinogen survival in 15 poorly controlled diabetic subjects, which was reversible with good control of blood glucose levels and also by infusion of heparin, suggesting that the action of thrombin was involved. Subsequently it was shown that the short fibrinogen survival was due to factors operative in the poorly controlled diabetic environment, as labelled fibrinogen obtained from poorly controlled diabetic patients had a normal turnover rate when infused into well controlled diabetic subjects (as reviewed by Jones and Petersen, 1981). Other studies have suggested that fibrinopeptide A levels are increased in diabetes indicating thrombin activation (Jones, 1985) but initial reports require confirmation. In diabetic nephropathy, increased excretion of fibrin degradation products in the urine has been reported (Chan et al, 1982), suggesting that intraglomerular thrombosis may contribute to renal damage.

Thus in diabetes there is some circumstantial but no conclusive evidence of an enhanced thrombogenic tendency with increased turnover of fibrinogen and deposition of fibrin. The interpretation of haemostatic function studies in diabetes mellitus is complex and the comparatively crude in vitro measurements of single factors probably bears little relationship to the intricate control of the haemostatic system in vivo but

the changes described support the existence of a thrombogenic tendency in diabetes mellitus.

## 1-7 PERIPHERAL BLOOD FLOW IN DIABETES MELLITUS.

### 1-7-1 Introduction

Blood flow is under the control of neural and hormonal systems, intrinsic autoregulatory capacity and the rheological properties of blood, all of which may be altered by the diabetic state. Disturbances of blood flow occur early in diabetes, but whether these changes represent an appropriate response to the raised metabolic rate and need for heat dissipation, or are manifestations of the effects of autonomic nerve dysfunction, the direct effects of hyperglycaemia and accompanying hormonal disturbances, or are due to alterations in the vessels has yet to be determined. These flow disturbances are suspected of leading to the development of vessel damage. In addition, haemorheological abnormalities, blood hypercoagulability and activation of the haemostatic mechanism, with release of vasoconstrictor substances and alteration of prostaglandins, may all affect the blood vessels and consequently blood flow.

### 1-7-2 The Early Studies of Peripheral Blood Flow in Diabetes Mellitus

Peripheral blood flow has classically been studied by venous occlusion plethysmography (\*as reviewed by Christensen, 1972). Interesting early studies, using less accurate equipment and techniques than are currently available, identified the presence of disturbed blood flow control in diabetic patients. Amongst the first studies were those made by \*Megibow and his colleagues in 1953, who used venous occlusion plethysmography to examine blood flow in the halluces of 47 diabetic patients. The response of halluceal blood flow to nitroglycerine and ganglionic blocking agents, which are both vasodilators, was examined. Vasodilation, to both agents, was impaired in diabetic patients and the authors thought that this represented occlusion of small vessels due to diabetic microangiopathy. However, although no patient in the Megibow study are reported as having evidence of peripheral vascular disease or pre-existing autonomic neuropathy, failure of vasodilation could also have been explained by the presence of either digital vascular disease or autonomic neuropathy. Methods for assessing the presence of mild neuropathy were not advanced in 1953. In the same year, a similar study with similar results and conclusions was reported by \*Mendlowitz et al, (1953). Mendlowitz and his colleagues used a calorimetry method to examine the effects of indirect heating and administration of a ganglionic blocking agent on circulation in the big toe. Nine out of the 38 diabetic patients, examined by the Mendlowitz group, showed impaired vasodilation, which was again interpreted by the authors as being a sign of an organic vascular lesion.

Soon afterwards evidence, of a possible neurogenic basis for the blood flow abnormalities described in diabetes mellitus, was provided by studies of skin temperature responses to indirect heating and cooling of the calf and thumb in diabetic patients (\*Martin, 1953). Twenty diabetic patients with symptoms and signs of neuropathy were examined. Skin temperature of the leg was abnormal in the majority of diabetic patients, 9 of whom initially demonstrated higher skin temperature and 10 lower skin temperature, than control subjects. The response of skin temperature to indirect cooling and heating was reported as being impaired. Patients with abnormally low foot skin temperatures were then given priscol, a drug which produces vasodilation whether the nerve supply is intact or not. A prompt rise of temperature in the patients occurred and was felt to demonstrate the presence of intact microvessels and probable neurogenic basis of the blood flow abnormalities in diabetes mellitus.

Various other studies of impaired temperature regulation in the diabetic limb have also been interpreted as manifestations of diabetic autonomic neuropathy. \*Sigroth, (1957) found that skin temperature changes of the fingers of diabetics to indirect heating was impaired in 53 out of 91 diabetic subjects and noted, that in some cases, these abnormalities were reversible with careful regulation of blood sugar levels. \*Aagaes & Moe, (1961) found impairment of temperature rise on indirect heating of the foot in association with the presence of vascular calcification and the presence of anhidrosis, findings both suggestive of autonomic neuropathy. (Calcification of vessels has recently been shown to be a feature of denervated limbs, [Goedel & Fuessl, 1983]). \*Weber and Wicht, (1962) examined the pulse volume changes in response to indirect heating in diabetics, without evidence of macrovascular arterial disease, and found that abnormal responders had more pronounced morphological changes in small vessels of the skin, suggesting that the state of the blood vessel was an important factor in determining blood flow.

Thus early evidence provided support for both the presence of abnormal neurogenic control mechanisms of blood flow and of an intrinsic vascular lesion in diabetes mellitus.

### 1-7-3 Evidence of Overperfusion in Diabetes Mellitus

At present, emphasis is being placed on the concept that high blood flow rates in diabetes mellitus might damage the vasculature. Increased resting blood flow in diabetic patients was noted by \*Alexander et al in 1968 who studied flow in forearm muscle. This finding was confirmed by Christensen (1970), who also found high flow rates in diabetic patients, particularly in association with poor diabetic control. Gundersen (1974), found that these high blood flow rates returned to normal with reversal of hyperglycaemia. In addition, the renal hyperfiltration of uncontrolled newly diagnosed diabetes is thought to be a manifestation of increased renal plasma

flow and, this too appears to be related to diabetic control (Parving et al, 1984). Also, increased retinal blood flow is a feature of diabetes, although with the development of severe retinopathy, flow rates diminish and ischaemia supervenes (Kohner et al, 1975). Thus, it is generally agreed that high blood flow rates occur in diabetic patients.

Blood flow to an organ or tissue is determined by the mean pressure gradient between the arteries and veins and by vascular resistance. During poor diabetic control, the arterial pressure is unchanged and venous pressure is low and constant. Thus the increased blood flow noted, often in combination with raised blood viscosity, suggests that vascular resistance is reduced and this could result in capillary overperfusion and in increased capillary hydrostatic pressure. This concept of overperfusion and capillary hypertension has been proposed as leading to enhanced leakage of plasma proteins and their deposition in the walls of arterioles and capillaries resulting in increased basement membrane thickness and alterations of the physical chemical properties (Parving et al, 1983[b]). There is some direct evidence to support this theory. Glomerular capillary pressure appears to be higher in moderately hyperglycaemic streptozocin diabetic rats compared with controls, (Hostetter et al, 1981), but in man direct estimates of capillary pressure have only been possible in nailfold capillaries. Whilst capillary pressure in young type 1 diabetic was not significantly different in the hand at heart level (Tooke, 1980), in the dependant foot capillary hypertension is demonstrable (Rayman et al, 1986). Whether or not high flow rates in diabetic patients are an appropriate physiological response to hyperglycaemia, rather than due to an intrinsic failure of flow autoregulation or autonomic neuropathy, there is some theoretical reason to believe that high blood flow might be harmful.

#### 1-7-4 The Development of Underperfusion

In the limbs, the development of atheroma may result in abnormally low resting peripheral flow rates and thus the pattern in diabetes mellitus may be one of initial "overperfusion" supervened by decreasing blood flow rates associated with long duration of diabetes mellitus and the development of vessel disease. The eventual development of low flow in vessels has also been attributed to the development of microvascular sclerosis and inherent stiffness of small vessels. However, a functional defective response of vessels to normal vasodilatory stimuli cannot be excluded.

The vascular response after a period of ischaemia is considered to be a sensitive measure of the state of the vessels and their capacity to dilate. In diabetic patients various abnormalities of reactive hyperaemia following arterial occlusion have been reported. First, the time taken for blood flow to reach its maximum value, after release of an arterial tourniquet which has induced ischaemia, is delayed (Haitas et al, [b] 1984) Second, the maximum peak blood flow value attained after a period of ischaemia is reduced, particularly in patients with longstanding diabetes (Christensen,

1969). Both these results suggest that vascular distensibility is decreased in diabetes but are apparently contradicted by Alexander et al, (1968) who reported that blood flow during reactive hyperaemia (in association with higher resting flow values) was greater in diabetic subjects than in normal subjects. However, Alexander and his colleagues found that the rise in integrated capillary pressure was lower and slower during reactive hyperaemia in diabetic subjects and suggested that a shunting of blood through arterio-venous anastomoses might be occurring at the expense of a decrease in nutritive blood flow.

#### 1-7-5 Evidence of Arterio-venous Shunting

The proposal of abnormal distribution of blood flow and of arterio-venous shunting in diabetes was first raised by Popoff in 1934 and is currently receiving renewed attention. Studies with Doppler probes using the ultrasound principle have shown abnormalities of the blood velocity waveform. In cases of diabetic neuropathy, the normal biphasic waveform with a small diastolic back flow is replaced by continued, rapid and increased forward flow in the feet (Edmonds et al, 1981). In diabetic neuropathic feet, the veins over the back are turgid and distended in the recumbent position and the venous oxygen tension is high (Boulton et al, 1982), suggesting that oxygen consumption is decreased. Although plethysmographic studies of arterial inflow typically reveal increased flow in poorly controlled diabetes, flow in the corresponding nailbed capillaries is lower than normal, (Tooke, 1983). These findings of Edmonds, Boulton and Tooke provide evidence for the presence of arteriovenous shunting of blood with by-passing of smaller nutritive channels. The cause of this shunting occurs has yet to be established, although it does appear to be a feature of neuropathic limbs.

#### 1-7-6 Autoregulation of Blood Flow in Diabetes Mellitus

Loss of appropriate autoregulation of capillary flow in response to changes in perfusion pressure has also been noted in diabetes. First, diabetic patients appear to be less able to maintain constancy of retinal blood flow in the face of changes of intraocular pressure (Sinclair et al, 1982); second, in the kidney, glomerular filtration rates diminish with reduction of blood pressure (and hence perfusion pressure) in diabetic patients with diabetic nephropathy (Parving et al, 1984); and third elevation by tilting results in an inappropriate fall of blood flow in the arm in diabetics, (Faris et al, 1982) whilst in the dependant foot (in diabetic neuropathy) flow rates remain higher than normal (Rayman et al, 1986). Whether these abnormalities are functional or structural has yet to be established.

#### 1-7-7 The Influence of Neural Control of Blood Flow

Blood vessels are richly supplied by sympathetic and parasympathetic nerve fibres. Nerve conduction times of the sympathetic and parasympathetic nervous system cannot

be tested directly but functional improvements of the autonomic nervous system reflexes occur with improvement of diabetic control (Hreidarsson, 1981). It has been postulated that reversible abnormalities of blood flow in diabetic patients might represent a transient autonomic nerve dysfunction associated with poorly controlled diabetes. Alternatively, delay of time to reach peak flow values and the reduction of maximal peak flow values could be explained by increased arterial rigidity.

#### 1-7-8 Summary

The presence of abnormal blood flow patterns in diabetes mellitus is well established, but the underlying basis of these disturbances has yet to be clearly elucidated. The development of permanent vascular and neurological damage obviously influences blood flow but, it is not entirely clear from previous studies, which of the disturbances of flow are functional and which are due to permanent damage. Nor is it clear whether early disturbances of blood flow are harmful or are, in fact, a necessary physiological adaptation to the diabetic state. High blood flow rates occur in diabetic patients but may be an appropriate response to the raised metabolic rate and need for heat dissipation. It is argued that high flow rates may result in capillary overperfusion and vascular damage; but paradoxically it has been suggested that much of this increased blood flow occurs through arteriovenous shunts, at the expense of nutritive blood flow, and that subsequent anoxia results in vascular damage and permanent hypoperfusion.

It seems feasible that reversible functional disturbances occur early in diabetic patients and that these result in loss of autoregulation of flow. Supervening structural and permanent vascular and neural damage will then further compound disturbances of blood flow. Disturbances of the neurovascular reflexes or metabolic effects acting locally on capillary sphincter tone remains a possible explanation of early alterations of blood flow. Alternatively, endothelial cell dysfunction secondary to the diabetic state, may affect the response of blood vessels to normal vasodilatory and vasoconstrictive stimuli. This may initially be reversible but lead to permanent damage. Also, a deterioration of the rheological properties of blood might contribute to impaired perfusion and preferential flow in larger channels of the small nutritive capillaries. The resulting anoxia may then result in more permanent damage to the vessel and tissues leading to the classical manifestations of microangiopathy and formation of new fragile vessels. Whilst this explanation is unlikely to be complete, it seems reasonable to assume that functional blood flow disturbances precede and contribute to the development of microangiopathy and that we do not yet know exactly how.

## 1-8 BIOSYNTHETIC HUMAN INSULIN

### 1-8-1 The Development of Biosynthetic Human Insulin

Apart from maintaining the blood glucose as close as possible to the normal range, no other treatment has been shown to protect against the development of diabetic microangiopathy and the long-term complications of diabetes. Until recently diabetic patients, dependent on insulin, have been treated by injection of insulin derived from beef or pork pancreases. Human insulin (biosynthetic) became available in 1979 when Goeddel and his co-workers announced the successful production of human insulin A- and B-chains through recombinant DNA technology using *Escherichia coli* fermentation. Small quantities of human insulin had previously been available from a variety of different sources including human cadaver pancreases, the fluid used to maintain pancreatic islet cells in culture and from chemical synthesis; but the amount of insulin obtained by these methods was insufficient for any widescale testing or clinical use. Shortly after the first testing of biosynthetic human insulin of recombinant DNA origin in man in 1980 (Keen et al, 1980) semi-synthetic human insulin, a product manufactured from porcine pancreatic insulin by trypsin catalyzed removal of the B30 alanine and its replacement by threonine (Markussen et al, 1983) also became available.

### 1-8-2 Structure and Properties of Human Insulin

The amino acid sequence of human insulin differs from that of porcine insulin by just one residue at the B30 position where the human B chain contains threonine and pork insulin alanine. Beef insulin is more different, containing alanine at B30, and also valine at position 10 in the A chain and alanine at A8 instead of isoleucine and threonine respectively. The physical differences of human insulin from pork insulin are slight, and no great differences in the biological action of human and pork insulins were anticipated. The safety and biological efficiency of human insulin were soon confirmed and found as regards blood glucose lowering effects and plasma insulin pharmacokinetics to be virtually identical to that of purified porcine insulin (see Skyler & Raptis, editors of Symposium on Biosynthetic Human Insulin 1981, Skyler, 1982, Karam & Etzwiler, editors of International Symposium on Human Insulin, 1983). However, even the small differences between human and porcine insulin result in slightly different properties. Human insulin is more quickly absorbed from subcutaneous injection sites, (Botterman et al 1981; Owens et al, 1984) a factor thought to be due to the greater hydrogen bonding capacity of B30 threonine on human insulin making it more soluble, (Chawdhury et al 1983). In practice, this results in quicker onset of action but consequently in a shorter overall duration of both short acting and intermediate depot insulin preparations. In clinical trials comparing diabetic control patients on twice daily subcutaneous injections of human and pork purified soluble and

isophane type insulins, overall glycaemic control has tended to be slightly worse on treatment with human insulin. In particular, fasting blood glucose concentrations have been higher on treatment with human insulin (Clarke et al, 1982 and as reviewed by Pickup, 1986). Manipulation of the timing of insulin injections or substitution of a longer acting insulin should allow comparable control to be achieved on human insulin and is of great practical importance.

### 1-8-3 Clinical Use of Biosynthetic Human Insulin

The major therapeutic advantage of human insulin appears to be that it is less immunogenic than purified porcine insulin which in turn is less immunogenic than beef insulin. In patients not previously treated with insulin, the production of circulating anti-insulin antibodies is less after treatment with biosynthetic human insulin than purified porcine insulin (Fineberg et al, 1983; Heding et al, 1984). The clinical importance of insulin antibodies is not fully understood. Lipoatrophy at insulin injection sites and substantial insulin resistance due to antibodies were largely a feature of treatment with beef insulin and are rare since the introduction of purified porcine insulins. Local and systemic allergic reactions to insulin are also rare but may be favourably treated by transfer to human insulin. However, it is possible that insulin antibodies may predispose to diabetic complications indirectly by affecting diabetic control. Whilst the amount and binding characteristics of anti-insulin antibodies have not been linked with the degree of metabolic control in individual patients, the presence of insulin antibodies in diabetic children has been associated with shortened 'honeymoon' remission period, higher insulin dosage and impaired endogenous insulin secretion. Indeed, insulin antibodies have the strongest relationship among various factors (these include HLA status, sex and age at onset) thought to influence residual B cell function. Insulin antibodies can prolong the intravenous half life of injected insulin. Patients with antibodies may show a delay in recovery from induced hypoglycaemia but on the other hand lose control less rapidly when insulin is withdrawn, suggesting that antibodies may protect from ketoacidosis (as reviewed by Pickup, 1986). Other postulated but not proven effects of insulin antibodies include a possible role in the development of microangiopathy and a suggestion that anti-insulin antibodies may cross-react with nerve growth factor and contribute to the development of autonomic neuropathy (as reviewed by Pickup, 1986).

Biosynthetic human insulin, being less immunogenic than animal insulins, is thus a justifiable choice of insulin for newly diagnosed insulin dependent diabetic patients and patients likely to require short-term or intermittent treatment, such as gestational diabetes or non-insulin dependent diabetic patients undergoing surgery. However, the role of human insulin in the treatment of established diabetics, particularly those with complications is not established. It is not known whether treatment with human insulin in the short or long-term would confer any advantage in this group of patients.



## 1-9 THE AIMS OF THE STUDIES PRESENTED IN THIS THESIS

Progress in the prevention of the vascular complications of diabetes mellitus requires a fuller understanding of the pathogenesis of diabetic vascular damage. The subject matter of this thesis consists of aspects of blood rheology and haemostatic function and of peripheral blood flow in diabetes mellitus. The aims of the studies are threefold.

First, in view of conflicting reports using disputable methods, to establish, using a dilute erythrocyte suspension filtration technique, whether a) a specific defect of erythrocyte deformability exists in diabetes mellitus and b) to define any relationship of impaired erythrocyte deformability to the presence of hyperglycaemia and insulin treatment.

Second, as the benefits of improving diabetic control in patients with pre-existing microvascular disease are now in question, to examine a) the relationship of control of blood glucose levels and the effects of improving diabetic control using intensified conventional multiple subcutaneous insulin regimes as can be achieved in normal clinical outpatient practice, on rheological factors, haemostatic function and peripheral blood flow in established diabetic subjects with evidence of microvascular disease and b) to monitor the effects of this regime on the progression of diabetic retinopathy.

Third, to establish whether treatment with biosynthetic human insulin, again administered by standard multiple subcutaneous injection regimes on an outpatient basis, produced any therapeutic advantage with regard to its effects on the haemostatic system, peripheral blood flow and peripheral nerve function in diabetes mellitus.

## CHAPTER 2

### RECRUITMENT OF PATIENTS AND STUDY PROTOCOLS

#### 2-1 RECRUITMENT OF DIABETIC PATIENTS

All the diabetic patients who took part in the studies presented in this thesis were recruited from the diabetic clinic at Leeds General Infirmary. Three groups of diabetic patients were recruited:

- a) newly diagnosed insulin-dependent diabetic patients presenting with weight loss and ketosis and also patients who had failed to obtain satisfactory control of blood glucose on treatment with oral hypoglycaemic drugs (see 2-1-1)
- b) established Type 1 and Type II diabetic subjects receiving treatment with either insulin or with oral hypoglycaemic drugs (see 2-1-2)
- c) established Type 1 diabetic subjects with evidence of microangiopathy on screening of the retinal vessels (see 2-1-3).

Patients from groups (a), (b) and (c) participated in the studies of the effects of improving diabetic control and in the studies of erythrocyte deformability. Only patients in group (c) participated in the double-blind crossover study of the effects of treatment with Biosynthetic Human Insulin (BHI) and Purified Porcine Insulin (PPI). Clinical diagnosis of Type 1 and Type II diabetes were assumed, as C peptide levels were not available on patients to provide confirmation.

##### 2-1-1 Newly Diagnosed Type 1 Diabetic Subjects and Oral Agent Failures

These were patients in whom the diagnosis of insulin-requiring diabetes mellitus had just been made. The author usually saw them within 24 hours of diagnosis. These patients participated in the studies of the effects of improved diabetic control on both haemostatic function and peripheral blood flow, and also in the studies of erythrocyte deformability.

The following selection criteria were applied:

- i) Newly diagnosed diabetes mellitus based on the World Health Organization criteria (1980, Second Report) with a history of weight loss and polyuria with findings of fasting whole blood glucose above 6.7 mmol/l or random blood glucose above 10.0 mmol/l, glycosuria and significant ketonuria. Also, patients previously treated with oral hypoglycaemic drugs, whose blood glucose levels remained continuously greater than 10 mmol/l and HbA<sub>1c</sub> levels more than

10% despite optimum treatment with diet and maximum doses of glibenclamide (20 mg) day and metformin (1500 mg day).

- ii) Age above 12 and under 70 years
- iii) Either sex
- iv) Generally active and in good physical health apart from diabetes mellitus

The exclusion criteria were:

- i) Physical or mental disability likely to hinder participation
- ii) Current malignant disease
- iii) Pregnancy
- iv) Drug treatment apart from oral hypoglycaemic drugs or antihypertensive agents

## 2-1-2 Established Type 1 (Insulin-Dependent) and Type II (Non-Insulin Dependent)

### Patients

This second group of diabetic patients participated in the studies of erythrocyte deformability. Some of this group also participated in the studies of the effects of improved blood glucose control or haemostatic function and peripheral blood flow.

The selection criteria for this group of subjects were as follows:

- i) The presence of diabetes mellitus as defined by the World Health Organization (1980, Second Report)

The exclusion criteria were the same as for group a.

## 2-1-3 Established Diabetic Patients with Evidence of Microvascular Disease

The third group of patients participated in the studies of the effects of improved blood glucose control and in the double-blind crossover study of treatment with biosynthetic human insulin and purified porcine insulin on haemostatic function, blood rheology and peripheral blood flow and peripheral nerve conduction times.

The selection criteria for this group of subjects were as follows:

- i) The presence of diabetes mellitus as defined by the World Health Organization (1980, Second Report)
- ii) Evidence of diabetic retinopathy on screening of the retina by a trained ophthalmologist
- iii) Age between 20 to 70 years
- iv) Either sex

The exclusion criteria were the same as for groups described in 2-1-1 and 2-1-2.

## 2-2 RECRUITMENT OF NORMAL VOLUNTEERS.

Normal volunteers for study were recruited from members of staff of the Department of Medicine at Leeds General Infirmary. Additional subjects were recruited through the Blood Transfusion Service. Medical histories and examinations were performed to ensure subjects were in good health and not on any medications. A note was made of alcohol and cigarette consumption. Verbal consent was obtained for the tests performed, which consisted of venesection and non-invasive measurements of blood flow by venous occlusion plethysmography and laser doppler flow-meter. These subjects were not fasted before venesection.

## 2-3 PROTOCOL OF THE TRIAL OF IMPROVED DIABETIC CONTROL

The purpose of the studies of improved diabetic control was to examine the effects of lowering blood glucose levels on:

- 1) blood rheology
- 2) haemostatic function
- 3) peripheral blood flow
- 4) peripheral nerve function.

These patients were studied on two occasions, 1) at entry to the study and 2) after a 12 week period of attempted improved diabetic control. A heterogeneous group of diabetic patients was examined including newly diagnosed Type 1 and established Type 1 and Type II diabetic patients. The object of the study was to improve diabetic control by attention to diet, patient education, the introduction of regular home glucose monitoring, intensive outpatient supervision and optimization of multiple daily subcutaneous insulin and oral hypoglycaemic regimes. In established insulin treated patients this period formed the run-in to a double-blind crossover study of treatment with BHI and PPI insulins (see Figure 2-1)

Patients participating in the studies had all procedures explained to them in detail by the author and gave informed signed consent. Diabetic care was managed by the author and patients were seen separately from the main diabetic clinic. The patients were examined clinically and weight and height were measured. Blood urea, electrolytes, liver function tests and haematology were checked and the urine was examined for proteinuria and haematuria. A note was made of alcohol and cigarette consumption. The hospital diabetic dietitian saw patients at entry to the study and made a record of the current dietary habits and supplied advice where necessary. Dietary allowances were appropriate to individual needs, and did not aim to achieve weight reduction.

Diabetic control was monitored by the patients own tests and by laboratory measurements. Patients were taught to use Ames glucometers and were required to keep regular records of capillary blood glucose levels. They performed fingerprick tests pre-prandially, one hour post-prandially and at bedtime and were asked to make a note of the time and frequency of any hypoglycaemic attacks. Blood tests were checked on three days of the week and urine daily. Patients treated with insulin were instructed to alter daily insulin dosages by up to 8 units, and to aim for fasting blood glucose levels of less than 7 mmol/l and one hour post-prandial blood glucose levels of less than 10 mmol/l. The patients were seen monthly to review their diabetic control and to take blood for HbA<sub>1</sub>C and laboratory whole blood glucose measurements. Adjustments in medication were made by the author at these visits. Additional clinic visits to provide support and advice were arranged as necessary. The newly diagnosed insulin dependent diabetic patients were initially managed as in-patients.

As radical improvement of blood glucose levels has been associated with paradoxical deterioration of diabetic microvascular disease, particularly in the eye, regular review of the patients' retinal state was carried out throughout the studies of improved blood glucose control. In the double-blind crossover study of insulin treatment, fluorescein angiograms of the retinal circulation were performed at the beginning and at the end of the study to assess whether any deterioration or improvement of the retinal state occurred in association with improvement of diabetic control.

#### 2-4 PROTOCOL OF THE DOUBLE-BLIND CROSSOVER STUDY OF THE EFFECTS OF TREATMENT WITH BIOSYNTHETIC HUMAN (BHI) AND PURIFIED PORCINE INSULINS (PPI).

The aim of the Double-Blind Crossover Study of BHI and PPI was to assess the effects of treatment with these insulins on:

- 1) blood rheology
- 2) haemostatic function
- 3) peripheral blood flow
- 4) peripheral nerve function
- 5) diabetic control.

The patients participating in these studies were managed as described in 2-2 and 2-3 (see Figure 2-1 for schematic representation of the protocol)

The factors under study were measured on four occasions:

- 1) the first, at entry to the study

# DOUBLE-BLIND STUDY OF TREATMENT

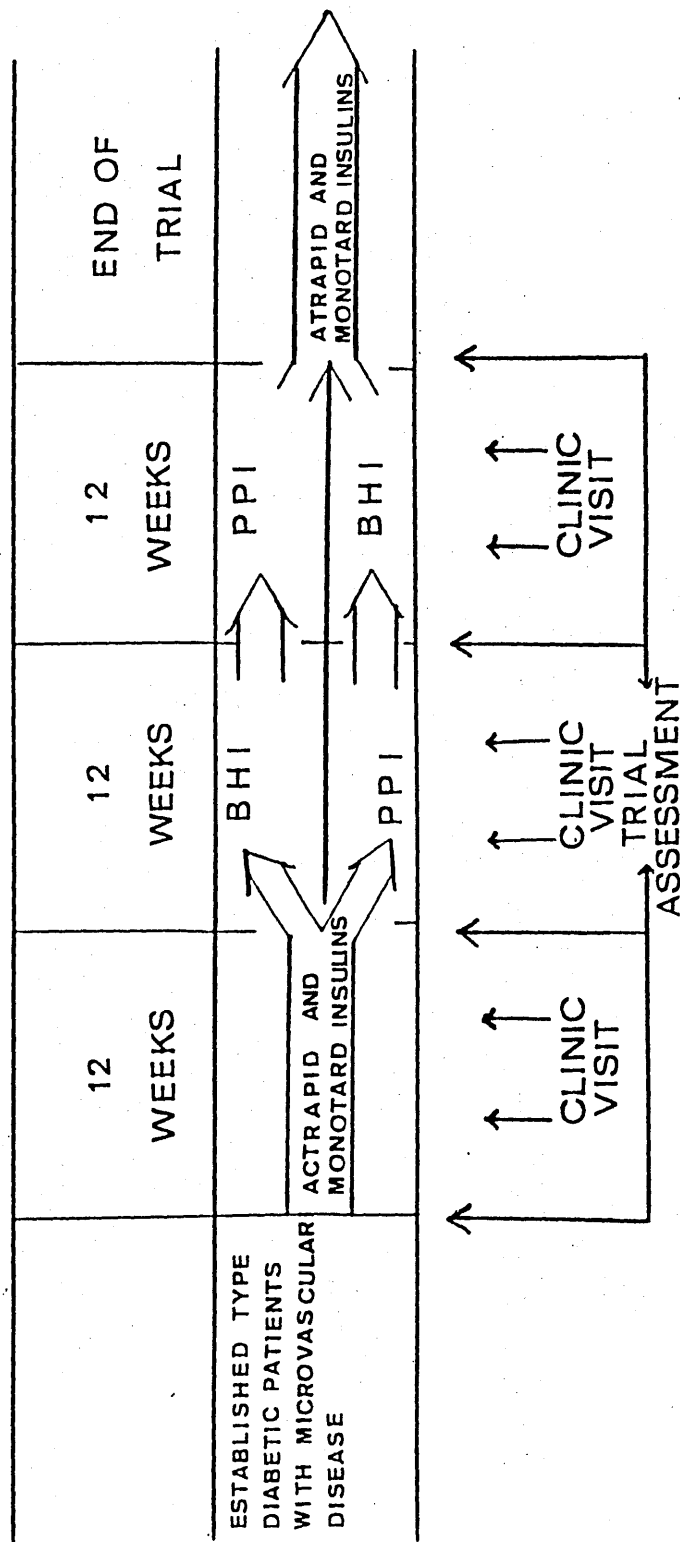


FIGURE 2-1

Protocol of the Double Blind Study of Treatment with BHI and PPI

- 2) the second, after a three month run-in period of attempted improved diabetic control on twice daily subcutaneous injections of quick and intermediate acting porcine insulins (actrapid and monotard)
- 3&4) the third and fourth occasions, after three months respective treatment with each study insulin.

The trial insulins were supplied by Eli Lilly Company labelled as insulin one and insulin two, with identical formulations of porcine and human soluble and isophane insulins. The sequence of allocation of insulin had been randomized by Eli Lilly and patients were entered sequentially on recruitment to the study, to receive the insulin prepared for patient number 1, patient number 2 etc. Sealed code cards were not broken until after completion of the study.

## 2-5 PROTOCOL OF THE STUDIES OF ERYTHROCYTE DEFORMABILITY

The aims of the studies of erythrocyte deformability were to determine whether there was a specific abnormality of erythrocyte deformability in diabetes mellitus and to examine the relationship of erythrocyte deformability to diabetic control. Patients participating in these studies were recruited from the diabetic clinic at Leeds General Infirmary. Verbal consent was obtained and patients were asked to attend for venesection in the early morning, fasting, before taking their usual medication. Blood for glucose and HbA<sub>1c</sub> levels was taken at the same time.

## 2-6 ETHICAL COMMITTEE APPROVAL

Local ethical committee approval was obtained for all the studies presented in this thesis.

## CHAPTER 3

## METHODS

## 3-1 DIABETIC CONTROL AND ROUTINE BIOCHEMISTRY, HAEMATOLOGY AND INSULIN LEVELS.

3-1-1 Whole Blood Glucose

This assay was performed by the laboratory staff of the chemical pathology laboratory of Leeds General Infirmary using a modification of Trinder's method. Blood for the assay was collected into Brunswick Fluoride/Oxalate tubes containing 3 mg of potassium oxalate and 1 mg of sodium fluoride.

3-1-2 Haemoglobin A<sub>1</sub>C

These were measured by Mr M Stickland of the Department of Medicine at Leeds General Infirmary using an Isoelectric Focussing Method (Stickland et al 1982).

Normal non-diabetic HbA<sub>1</sub>C levels ranged from 5.4-7.2% and the intra-assay Coefficient of Variation (CV) for measurements was 2.8%.

3-1-3 Urea and Electrolytes and Liver Function Tests

These were measured by the chemical pathology laboratory at Leeds General Infirmary using an automated SMA II analyzer.

3-1-4 Full Blood Counts

These were measured by the haematology laboratory in Leeds General Infirmary using a Coulter S Plus Counter.

3-1-5 Insulin Assay

Plasma-free insulin levels were measured by Mr M Stickland of the Department of Medicine of Leeds General Infirmary. Blood for the assay was collected into lithium heparin tubes and plasma was separated off and stored at  $-30^{\circ}\text{C}$  until the time of measurement. All the samples were assayed on the same run. Insulin was measured using a radioimmunoassay kit supplied by the Immuno Nuclear Corporation, Stillwater, Minnesota. The option A assay procedure was followed. The samples were treated with PEG before the assay procedure to precipitate out insulin antibody-bound insulin. Guinea pig anti-insulin serum (first antibody) and Iodine<sup>125</sup>/insulin (tracer) were then added to the specimens and incubated for 16-20 hours at  $2-8^{\circ}\text{C}$ . A pre-precipitated second antibody complex (rabbit anti-guinea pig precipitating complex)



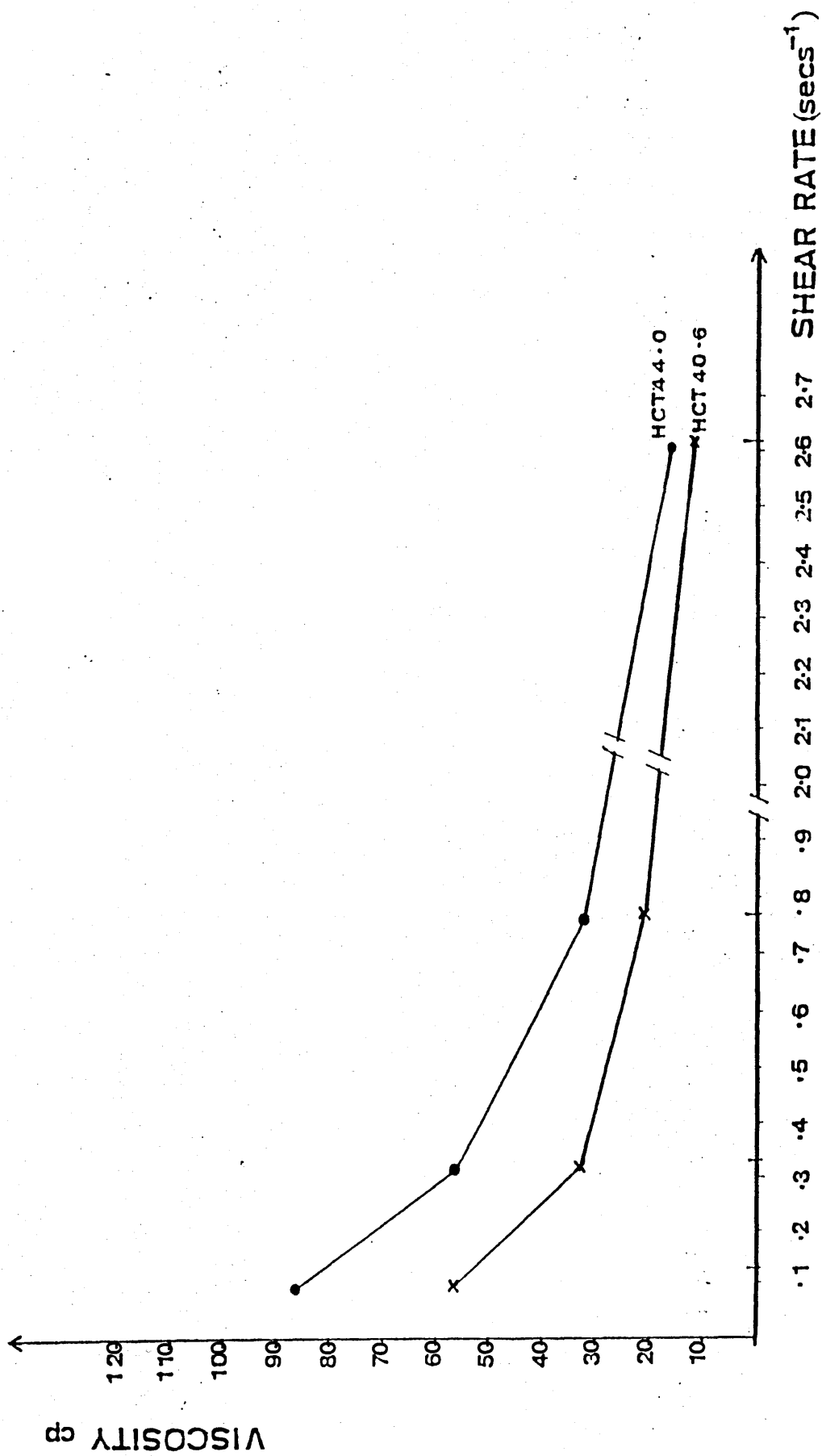


FIGURE 3-1

Graph of Whole Blood Viscosity Measured at Different Shear Rates

was added to the specimens which were centrifuged and decanted, after 15-25 minutes incubation at 20-25°C, and finally the precipitate was counted in a gamma scintillation counter. Normal insulin levels for the kit assay were less than 25 u/ml and the intra-assay CV was 5.3%. Insulin standard, zero standards, non-specific binding standards and total count tubes were run along with the test specimens.

### 3-2 WHOLE BLOOD VISCOSITY

#### 3-2-1 Introduction

Measurements of whole blood viscosity were made by the author using the Contraves Low Shear 100 viscometer (Contraves Instruments Ltd, Ruislip, England) at shear rates of 2.62  $\text{secs}^{-1}$  and 0.130  $\text{secs}^{-1}$ . Viscosity is a measure of the ease with which flow occurs in a fluid in response to an applied force. This concept was suggested by Newton in 1713 (see Usami, 1982) and simple fluids which flow proportionally faster when more force is applied, maintaining a constant viscosity, are called Newtonian. Whole blood is a non-Newtonian fluid (Dormandy, 1970); its viscosity is greater at low flow rates and decreases with increasing flow rates. This property is demonstrated in Figure 3-1 which shows whole blood viscosity in a diabetic patient (measured in the Contraves Low Shear Rate Viscometer) plotted against a range of different shear rates. Each point in Figure 3-1 represents the mean of single measurements from 3 separate aliquots of blood.

#### 3-2-2 Determinants of Whole Blood Viscosity

The main determinants of whole blood viscosity are the plasma proteins, the erythrocytes and other cellular elements and the temperature of the sample at the time of analysis (as reviewed by Merrill, 1969 and by Stuart & Kenny, 1980). Numerically, erythrocytes are the most important of the cellular elements of blood and viscosity increases with increasing haematocrit (Begg & Hearn, 1966). This property is also demonstrated in Figure 3-1, which shows the effects of shear rate on whole blood viscosity. A second blood sample obtained from the same individual at a later date, with a slightly lower haematocrit, is less viscous at any given measuring shear rate. Leucocytes are more resistant to flow than the erythrocytes but being present in much smaller numbers are less important except where the number is pathologically raised (Lichtman, 1973).

The tendency of erythrocytes to aggregate at low flow rates and to deform and to align at higher flow rates accounts for the non-Newtonian properties of whole blood (see Merrill, 1969). As with other non-Newtonian fluids a minimum force has to be applied to blood to initiate flow. This force is known as the yield stress (see Merrill, 1969; Kiesvetter et al, 1982). Thus in order to obtain meaningful measurements of the viscosity of non-Newtonian fluids, such as whole blood, standard conditions with

defined forces or flow rates must be applied. Samples have to be measured at a constant temperature and the haematocrit of the sample has to be taken into account.

### 3-2-3 Contraves Viscometer

Whole blood viscosity was measured in the Contraves Low Shear 100 viscometer (Contraves Instruments Ltd, Ruislip, England) at shear rates of  $2.62 \text{ secs}^{-1}$  and  $0.130 \text{ secs}^{-1}$ . A diagram of the instrument is shown in Figure 3-2. The Contraves is a rotational viscometer which operates on the same principle as the Wells Brookfield. A stainless steel bob is suspended by a wire into a stainless steel specimen cup which is kept at a constant temperature by a water jacket connected to a thermostatically controlled water bath. The cup is rotated by a geared electric motor at different speeds on shear rates of  $0.1 - 5.0 \text{ secs}^{-1}$  in 15 stages. The torque applied to the bob is transmitted to the wire which then tends to twist. A photoelectric mechanism, detects the twist of the wire and controls an electromagnet which supplies an equal and opposing torque to prevent movement. The current from the electromagnet is proportional to the shear stress. A graph of shear stress against time was recorded by a chart recorder. An example is shown in Figure 3-3. This demonstrates the typical initial peak on applying shear, followed by a slow fall of the measured shear stress with time. These phenomena are thought to represent the thixotropic properties of blood caused by disaggregation of the red cells during the period of measurement (Stuart and Kenny, 1980). Viscosity was measured 24 seconds after commencing shear as this was found to be the most consistent point (Milligan, 1983 [b]). The coefficient of variation (CV) for 11 consecutive measurements was 3.2% at shear rate  $2.62 \text{ secs}^{-1}$  but the lower shear rate of  $0.130 \text{ secs}^{-1}$  showed a much wider variation in CV from 3.8-11.2%, see table 3-1.

### 3-2-4 Protocol for Measurements of Whole Blood Viscosity

Whole blood for viscosity measurements was collected into ethylene diamine tetra acetic acid 4m.mol/L (EDTA) from fasting patients and mixed on a roller mixer prior to measurement. The measurements were performed within three hours of venesection. The viscometer was placed on a solid weigh bench to reduce vibration and a perspex shield was placed round the apparatus to prevent draughts. The instrument was zeroed on air and then the calibration checked with a standard oil (7.78 mPa's at  $37^{\circ}\text{C}$ , Brookfield Engineering Inc, Stoughton, Ma. USA). Using an automated pipette, 0.35 ml of sample was placed in the cup, taking care to avoid bubbles, and the bob was lowered into the sample. Equilibration of temperature occurred rapidly. The chart recorder was started, then the drive to the viscometer was switched on at the pre-determined rate. Measurements of whole blood were made in triplicate and the results expressed as means.

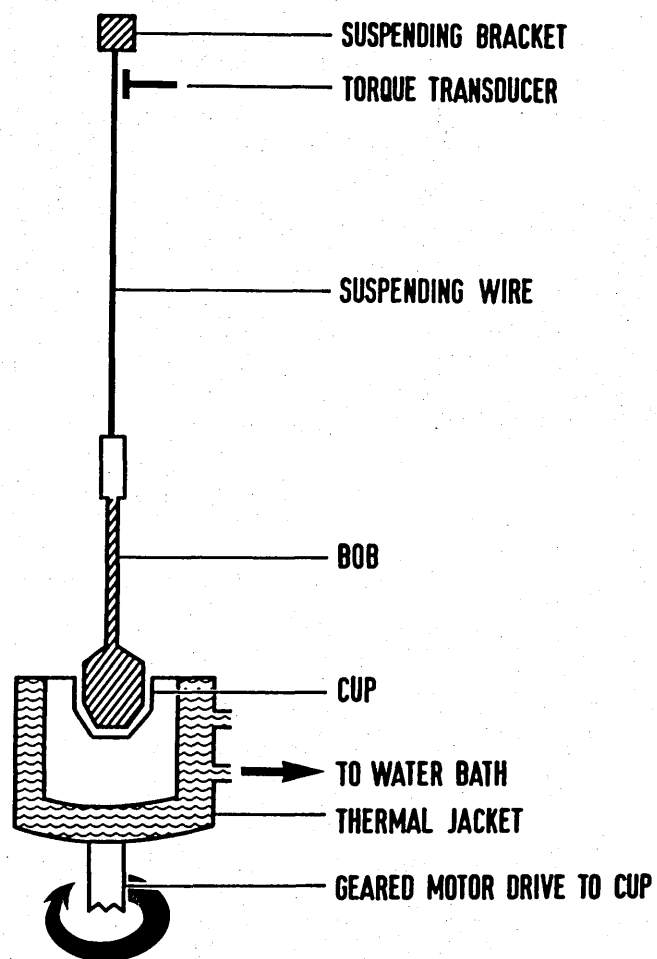
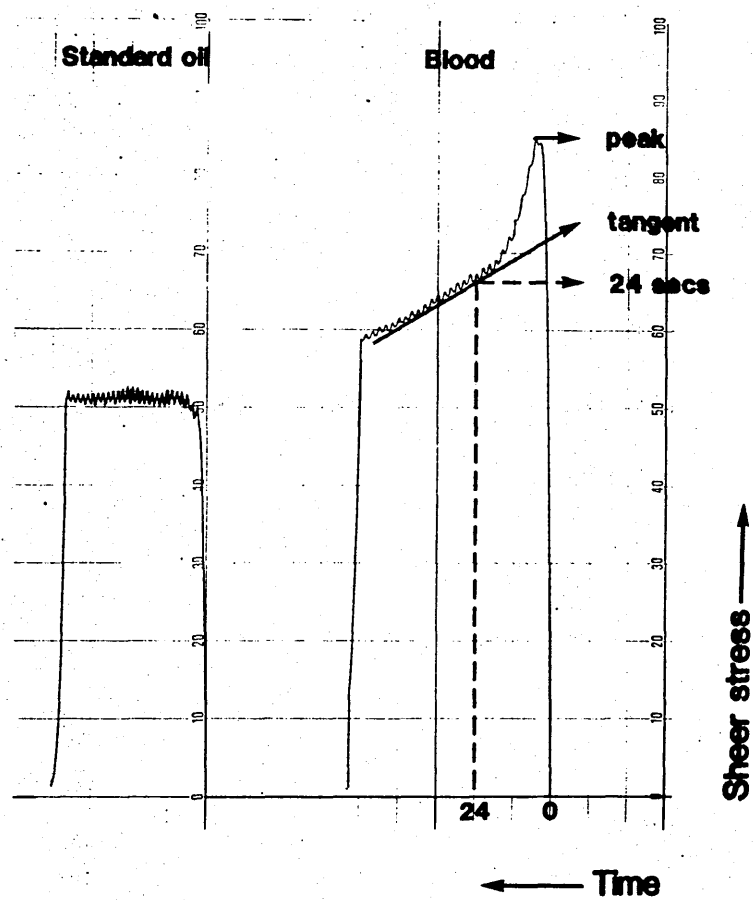


FIGURE 3-2

The Contraves Low Shear Rate Viscometer



**FIGURE 3-3**

Example of Graph Recording of Shear Stress against Time used for Calculating Whole Blood Viscosity

### 3-3 ERYTHROCYTE DEFORMABILITY

#### 3-3-1 Introduction

Measurements of erythrocyte deformability were made by the author. Erythrocyte deformability is the ability of erythrocytes to change shape under fluid shear and it is this property which accounts for the low viscosity of blood during flow despite its high cellular content. Deformability has been assessed using a variety of techniques including 1) the micropipetting of cells, 2) subjecting cells to fluid shear and observing the change in shape, 3) filtration techniques, 4) centrifuge techniques and 5) by direct examination of membrane fluidity - although it is not clear how these very different techniques are related qualitatively and quantitatively.

#### 3-3-2 The Erythrocyte Filtration Technique

For the experiments presented in this thesis, deformability was measured indirectly by an erythrocyte filtration technique. Erythrocytes are passed through filters which are micropore sieves having pores of similar diameter to the smaller nutritive capillaries. Pore sizes of 3-5 microns, the size of the smallest capillaries in the body, are considered suitable (Chien, 1981). The erythrocyte has a diameter of about 7 microns and, in order to pass through the filter, it has to change shape. Deformability is measured as the ease with which erythrocytes pass through the filter, either by recording the volume of cells filtered under the influence of a fixed force (Schmid-Schonbein, 1973), or conversely, the pressure which develops on fixed rate filtration (Kenny et al, 1981).

The first erythrocyte filtration experiments were by Protheroe and Burton in 1962, using filters manufactured by Millipore. The quality of these coarse surfaced filters, whose pores are angulated, irregular, of variable diameter and variably placed, made standardized measurements difficult. The introduction of more uniform polycarbonate filters by Gregerson et al, (1967) allowed more reliable study of erythrocyte filtration. Even so, inter-batch variation between these filters has led to recommendations that filters from the same batch should be used in comparison studies. Other filter materials, such as reusable silver membranes (Baar, 1976), have been used but most filtration studies have used polycarbonate Nucleopore filters (Pleasanton Corporation, USA) (Schmid-Schonbein et al, 1973; Usami et al, 1975; Reid et al, [b] 1976; Barnes et al, 1977; Juhan et al, 1981).

#### 3-3-3 Factors Influencing Erythrocyte Filtration

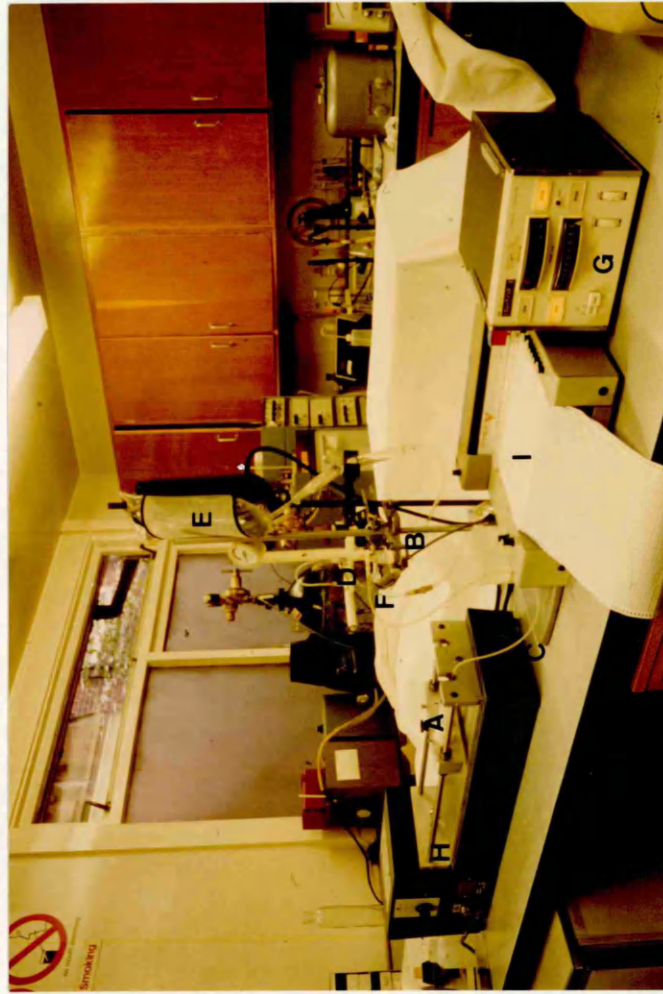
Filtration studies have been performed on both whole blood and on erythrocytes in suspension. It is recognized that different factors influence the outcome in each situation. Whole blood filtration is affected by haematocrit, plasma proteins and lipids, erythrocyte aggregation and plugging of the filter pores by platelet aggregates and

leucocytes (Boisseau, 1981). Anticoagulants which chelate calcium, such as EDTA, theoretically minimize the platelet and leucocyte effects and are generally recommended (Lucas et al, 1983 [a]). However others have suggested that calcium levels may affect membrane fluidity (Mitkadu and Shuji, 1982). Despite these drawbacks, whole blood filtration is considered by some being more representative of erythrocyte flow in vivo, although it is not a specific test of erythrocyte function.

The study of erythrocyte suspensions permits standardization of both the haematocrit and suspending medium; preparing washed erythrocyte suspensions largely removes the potential leucocyte and platelet plugging effects and, in dilute suspension in buffer, the problem of erythrocyte aggregation is overcome. The disadvantages are that preparation of the cell suspensions may remove a sub-population of cells with reduced deformability. Indeed, it has been shown that washing of erythrocytes affects deformability by altering extra membranous factors (Rampling and Sirs, 1972). Filtration techniques provide an integral assessment of the cell population, and little information about the specific deformability of individual cells. Despite these criticisms, the study of dilute erythrocyte suspensions is now recommended for the measurement of erythrocyte filterability (Boisseau, 1981). Low pressure filtration, with forces approaching those to which the erythrocyte is subjected to in the capillary circulation, is felt to be a physiological test of deformability (Schmid-Schonbein et al, 1973), (although abrupt entry to a capillary sized channel does not mimic the tapering of the vasculature).

#### 3-3-4 The Erythrocyte Filtration Apparatus

In this study, erythrocyte filterability was measured by the washed-erythrocyte, positive-pressure method developed by Kenny and Stuart (1981) as adapted by Milligan (1983 [a]). This method complies with the criteria discussed above for accurate assessment of erythrocyte filterability. A photograph of the apparatus is shown in Figure 3-4, and is labelled as described. A 20 ml syringe (a) containing a dilute erythrocyte suspension ( $0.1 \times 10^{12}$  cells/l) is attached via a three-way connection to (b) a 'Pop-Top' filter holder containing a 5 micron pore size polycarbonate membrane filter (Nucleopore Corporation, Pleasanton, California). Fine bore pressure tubing of 1-2 mm diameter (c) connects a part of the three-way connection to (d) an "Intra flo" continuous flushing device attached to (e) a pack of isotonic saline held within a pressure infuser. The "Intra-flo" continuously releases a small amount of fluid which prevents contamination of the system with erythrocytes. Pressure changes occurring over the nucleopore filter were measured by (f) a Hewlett-Packard venous pressure transducer which was calibrated electronically to read between 0-20 mm Hg and (g) amplifier. The syringe is driven at a fixed rate by (h) a Harvard pump and the pressure rise with time is recorded on (i) a chart recorder. The accuracy of the transducer was checked periodically by a mercury manometer. In the Stuart and



(a)

20 ml syringe with erythrocyte suspension

(b)

'Pop-Top' filter holder containing a 5 m pore size polycarbonate membrane filter

(c)

fine bore pressure tubing of 1-2 mm diameter

(d)

'Intra flo' continuous flushing device

(e)

pack of isotonic saline held within a pressure infusor

(f)

Hewlett-Packard venous pressure transducer

(g)

amplifier

(h)

Havard pump

(i)

chart recorder

FIGURE 3-4

The Erythrocyte Filtration Apparatus



Kenny method, filterability was measured as the ratio of the area under the time-pressure graph obtained on filtering buffer, to that obtained on filtering cell suspension. In the Milligan adaptation, filterability was measured as the rate of rise of pressure between 06-66 seconds as this was found to be consistent parameter.

### 3-3-5 Protocol for Measurements of Erythrocyte Filtration

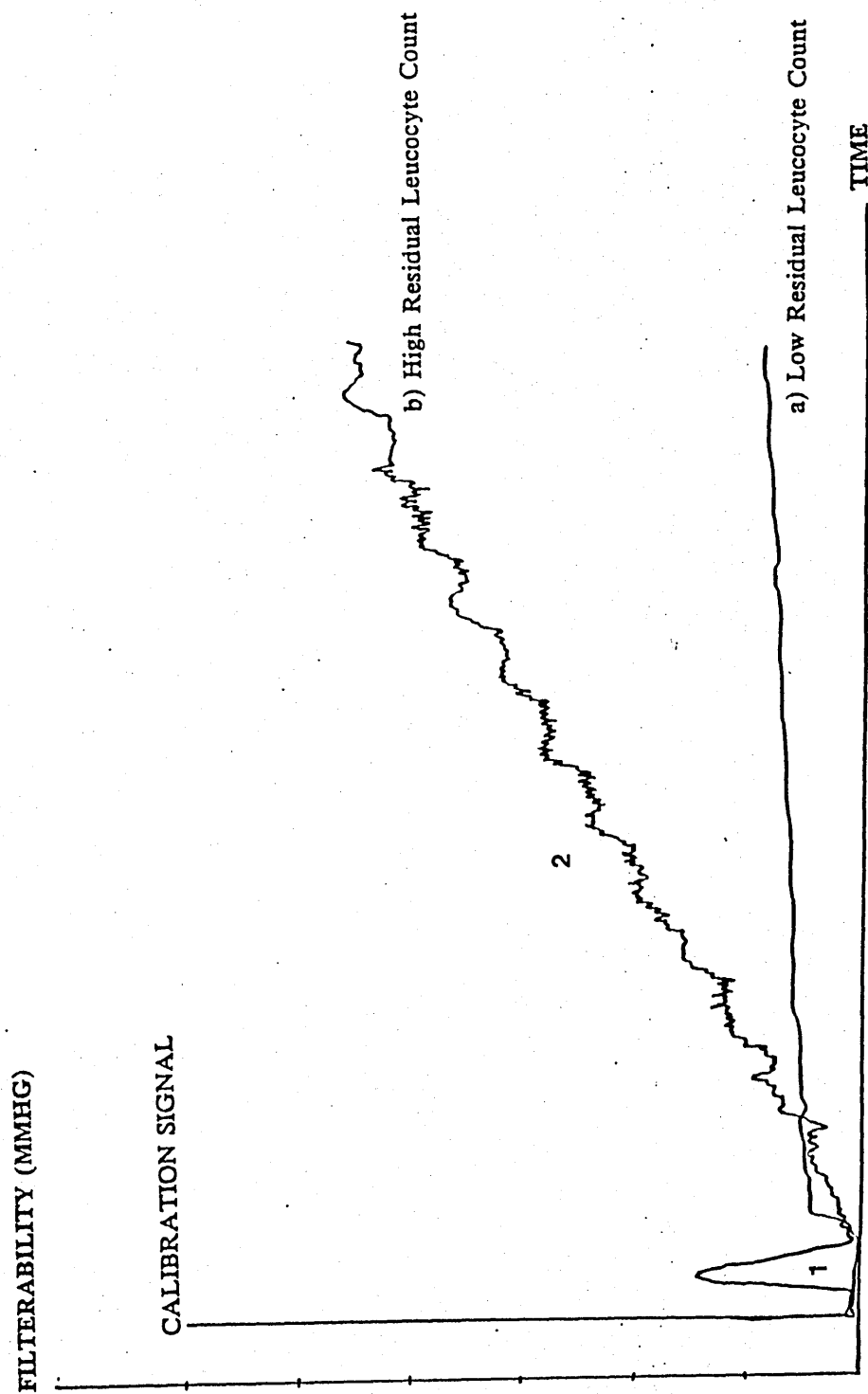
Blood samples for measurement of erythrocyte filterability were collected in 4 mmol/L EDTA and measurements of deformability were made within three hours of venesection. The cell count of the sample was estimated on a Coulter S Plus Counter. In most of the experiments, the anticoagulated whole blood was then pre-filtered over a cotton wool leucocyte removal filter (Imugard Leucocyte Removal Filter, Termuno Corporation, Japan) as follows: 2-3 mm filter material was packed in the barrel of a 5 ml syringe and soaked with phosphate-buffered saline after which 3 mls of whole blood was placed in the barrel of the syringe and allowed to drip into a collecting chamber. Residual erythrocytes were washed through the filter material by placing a further 3 mls of phosphate-buffered saline over the used filter material.

The filtrate was washed 3 times in PBS by centrifugation in a bench centrifuge at 2000 rpm, residual buffy layer and supernatant were aspirated and the erythrocytes were re-suspended in PBS. The suspension cell count was then rechecked in the Coulter S Plus Counter. This method removed almost all the leucocytes and all the platelets.

The washed erythrocytes were then made up to a concentration of  $1.0 \times 10^{12}/l$  and the residual concentration of the leucocytes was calculated from the dilution factor. The cell measurements of suspension were then drawn up in a 20 ml syringe and placed in the filtration apparatus. The pump was operated to fill the top pop holder with suspension and to wet the surface of the nucleopore filter. A stable baseline was obtained and the pump was then started at a rate of 3.5 ml/minute and the rise in pressure on filtration recorded on the chart. The resulting graph of pressure against time (see Figure 3-5) showed (1) a variable initial deflection on commencing filtration followed by (2) a rise in pressure against time. The initial deflection was excluded from measurements of filterability which was therefore taken as the rise of pressure occurring from 6-66 seconds of filtration. Figure 3-5 shows examples of erythrocyte suspension filtration recordings: (a) with a low residual leucocyte count showing a slow rise in pressure against time and sample (b) with a high residual leucocyte count showing a sharper rise of pressure against time. Effects of residual leucocytes on the measurement of erythrocyte filtration are discussed in detail in chapter 4.

### 3-3-6 Intra-Assay Coefficient of Variation of Erythrocyte Suspension Measurement

The initial pressure deflection, on commencing filtration of suspensions, is recommended by some as the most sensitive measure of erythrocyte deformability



**FIGURE 3-5**

Examples of Graph Recordings of Pressure against Time obtained on Filtration of  
Erythrocyte Suspensions

(Skalak et al, 1983). In the system used by the author, the mechanical artifact of starting the pump produced a large intra-assay CV of over 34%. The intra-assay CV for 7 consecutive measurements of the pressure rise from 6-66 seconds of starting the pump, (excluding the initial pressure deflection which occurs on starting the system, see figure 3-5) was 10.5% (see Table 3-2)

### 3-3-7 Inter Assay Variation of Erythrocyte Suspension Measurement

Erythrocyte suspension filterability was measured on blood samples collected from 2 normal non-diabetic subjects on 3 separate week days. Filterability of the suspensions was found to vary greatly and in subject (A) ranged from 1.1 - 3.5 mm Hg and in subject (B) from 1.0 - 9.0 mm Hg. It was noted, that, in each individual the sample containing the highest calculated number of residual leucocytes was the least filterable (see Table 3-2). This suggested that part of the variation in filterability could have been caused by differences in the number of leucocytes contaminating the suspensions on each occasion.

### 3-3-8 The Influence of the Erythrocyte Suspension Medium and the Sensitivity of the Erythrocyte Filtration System to the presence of Hardened Erythrocytes

#### a) Buffer

Most filtration studies now employ EDTA for whole blood anticoagulation and isotonic phosphate-buffered saline as the suspending medium. Phosphate-buffered saline (PBS) was used as a buffer in the erythrocyte filtration experiments unless otherwise stated. It was prepared fresh as required from commercial tablets of ion concentrate (Dulbecco A, Oxoid Company). The buffer contains NaCl 8 gm/litre, KCl 0.2gm/litre,  $\text{NaHPO}_4$  1.15 gm/litre and  $\text{KH}_2\text{PO}_4$  0.2gm/litre. Measurements of pH and of Na and K concentration were made, using a pH meter and the automated SMA II analyzer (see section 3-1), to check the quality of the buffer made up from ion concentrate tablets. The buffer pH was 7.4, Na content 152 mmol/l and K content 4.1 mmol/l. The osmolality of PBS was also checked and was found to vary from 285 - 291 mosmol  $\text{kg}^{-1}$ .

In order to assess the integrity of the erythrocytes prepared for the filtration experiments, the intracellular Na and K contents of erythrocytes, washed in PBS and passed over the leucocyte removal filter, were checked by flame photometry. These measurements were made by the Department of Chemical Pathology at Leeds General Infirmary. Loss of intracellular K and rise of intracellular Na is an indication of impairment of the cell membrane Na - K pump and the overall integrity of the cell membrane. The erythrocytes showed a small rise in intracellular Na and a fall in intracellular K, (see table 3-3). The magnitude of these changes, which were within

the normal intra-assay coefficient of variation, were not considered to be significant and thus the processed erythrocytes were considered functionally intact.

PBS made up from Dulbecco tablets does not contain calcium, unless this is added separately. In experiments of the effects of insulin on erythrocyte filtration, Tyrodes solution was used as a buffer, as calcium may be necessary for insulin to bind to cell membranes. Tyrodes buffer contains NaCl 8.0 gms/l, KCl 0.2 gms/l,  $\text{NaH}_2\text{PO}_4\text{H}$  0.05 gms/l, CaCl 0.2gms/l, MgCl 0.047 gms/l and glucose 1.0 gms/l. In practice, Tyrodes buffer was found to contain Na 153 mmol/l, K 2.8 mmol/l and the osmolality was 291 mosmol.kg<sup>-1</sup>. Tyrodes is a weaker buffer than PBS as it contains less phosphate. The pH was 7.3, but as addition of cells, in 3 separate samples, was found to result in an average rise of pH 0.38, Tyrodes solution for filtration experiments was prepared at a pH of 7.0.

#### b) Protein

The addition of albumin to buffers has been recommended to prevent cell crenation (Talstad, 1971). The filterability of cells in PBS with added bovine albumin 0.5% was 30.0% less than that of cells suspended in plain PBS. The concentration of albumin (5 gm/l) was higher than usual physiological levels and the interaction with cells may have blocked the Nucleopore filter pores. Therefore albumin was not added routinely.

#### c) Glucose

The effects of glucose were examined by comparing filterability of erythrocyte suspensions containing different concentrations of added glucose: 0 mmol/l, 5.0 mmol/l, 10.0 mmol/l, 20.0 mmol/l and 30.0 mmol/l. Results are shown in Table 3-4. There were no statistically significant effects of the presence of glucose in the suspending medium on suspension filterability. Glucose was not added to buffers used in the control and diabetic patient studies.

#### d) pH

Lowering the pH from 7.0 to 5.0 did not cause deterioration of filterability (n = 3) but the cells were not incubated for any length of time.

#### e) Gluteraldehyde

To check whether the filtration system could detect the presence of less deformable erythrocytes, the filterability of gluteraldehyde hardened erythrocytes was checked. Gluteraldehyde treatment hardens the erythrocyte membrane by crosslinking proteins. When erythrocyte suspensions were treated with 1.0% gluteraldehyde, no cells passed through the 5 micron Nucleopore filters and the filtration pressure rose rapidly to the

maximum recordable. This indicates that only erythrocytes capable of membrane deformation are able to pass through the 5 micron Nucleopore filter.

### 3-3-9 Changes in the Cellular Composition of the Erythrocyte Suspension and Destruction of Cells following Treatment with Leucocyte Removal Filter.

It is possible that the passage of erythrocytes over a leucocyte removal filter and the repeated washing of cells might result in the destruction of the least deformable cells, thus invalidating subsequent measurements of erythrocyte filterability.

#### a) Changes in Erythrocyte MCV.

To check for changes in the erythrocyte population during preparation of test suspensions the mean corpuscular volume (MCV) of cells was checked after three different methods of preparation:

- 1) washing the cells 3 times in PBS (as described above)
- 2) after filtering whole blood through Imugard Leucocyte Removal Filter (as described above)
- 3) after both processes combined, filtration first followed by washing

Results are given in Table 3-5. After washing, the mean  $\pm$  SD MCV of 8 samples of erythrocytes was  $87.9 \pm 4.4$  compared with the mean  $\pm$  SD MCV of  $88.1 \pm 4.2$  in whole blood ( $p=0.06$ ). The mean  $\pm$  SD MCV of these same erythrocytes when filtered was  $87.6 \pm 4.5$  ( $p=0.263$ ). After combined washing and filtering of 13 different blood samples, the mean  $\pm$  SD MCV was  $90.8 \pm 5.2$  compared with a mean  $\pm$  SD whole blood MCV of  $90.5 \pm 5.3$ , [ $p=0.153$ ]]. Thus preparation erythrocyte suspensions by washing and pre-filtering of blood over leucocyte removal filter did not appear to alter the composition of the erythrocyte population of blood samples.

#### b) Destruction of Erythrocytes

Haemolysis is the destruction of erythrocytes and thus any haemolysis occurring during the processing of erythrocytes is also an indication of loss of potentially less deformable cells. Visible haemolysis in the supernatant of spun cells occurred rarely and the blood sample was discarded. Light spectrophotometry, at a light emission of 415 nanometres, was used to detect if there was any minor haemolysis.

The method used was as follows. First, the light reflectance of standards of lysed solutions of known concentrations of erythrocytes in PBS were measured (see Figure 3-5A (1)). Second, the background light reflectance of the sample plasma at various dilutions with saline was measured (see Figure 3-5A, (2)). It is necessary to take the dilution of the test sample with PBS into account, as plasma gives a background light reflectance reading that varies directly with its dilution, whereas saline and PBS read zero. During the filtration procedure, the leucocyte removal filter was pre-moistened

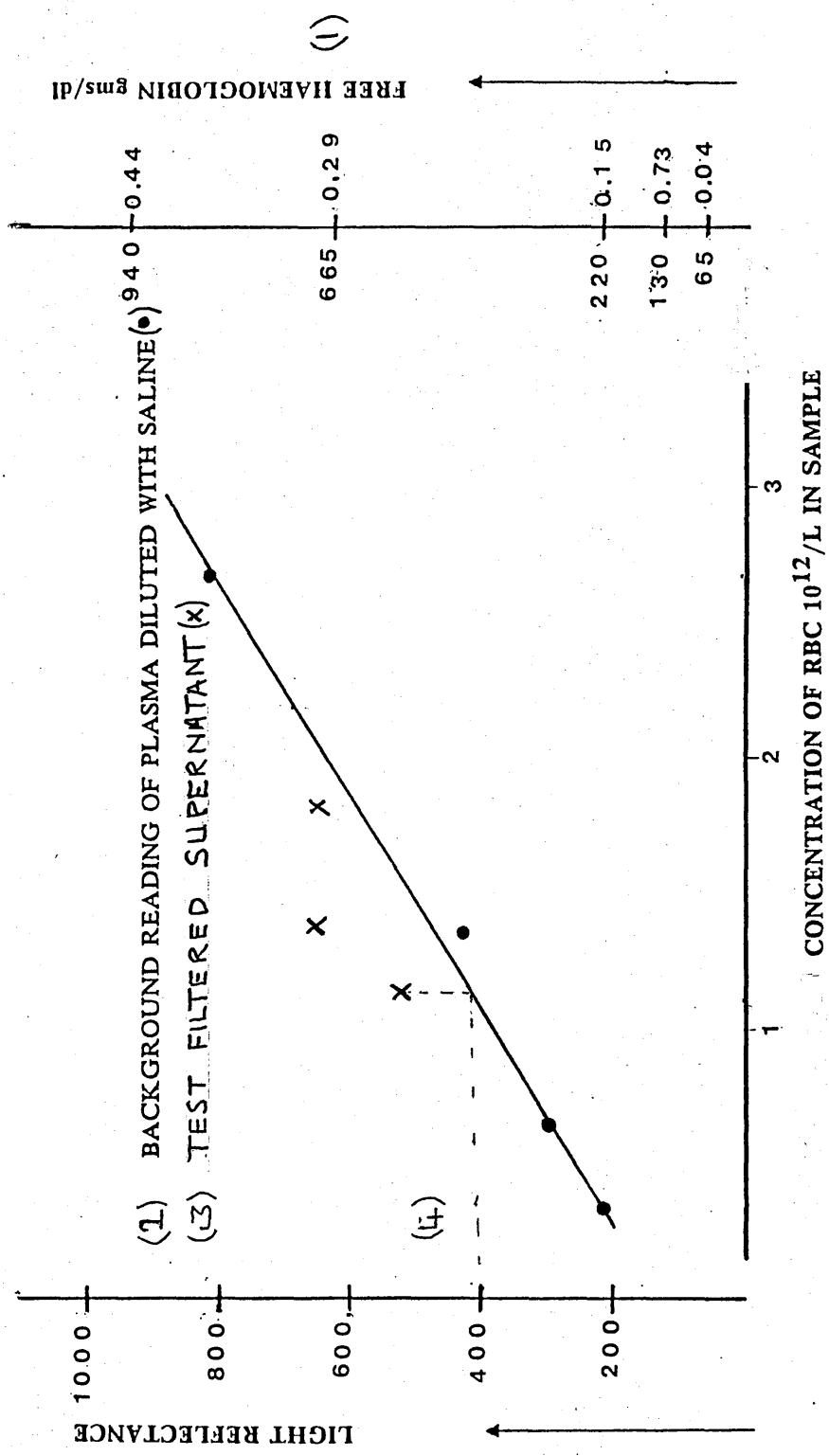


FIGURE 3-5A

Graph of Light Spectrophotometric Measurement of Plasma and Haemoglobin Solutions

with PBS and residual erythrocytes were flushed through with more PBS resulting in considerable dilution. Third, the light reflectance of the supernatant of test filtered blood samples was measured (Figure 3-5A, (3)).

The erythrocyte concentration of background and test samples, before separation of the supernatant, was measured on a Coulter S plus counter to give an estimate of dilution. Light reflectance results were plotted on a graph with the erythrocyte concentration on the x axis and the light reflectance value of samples on the y axis (see Figure 3-5A). The background light reflectance of the test samples due to plasma was calculated by extrapolation (see Figure 3-5A (4)) and subtracted from the test reading. The difference between test and background readings was due to haemolysis and the amount of free haemoglobin was calculated by reading off a graph of light reflectance of standard lysed solutions of haemoglobin, (Figure 3-5 A (1)).

Free haemoglobin in the supernatant of cells passed through the leucocyte removal filter was measured on 4 blood samples treated with leucocyte removal filter, as described above. Three separate test filtration measurements and a background plasma dilution graph were measured on each whole blood sample and light reflectance readings were made in duplicate. The mean  $\pm$  SD amount of free haemoglobin of 4 separate blood samples filtered over leucocyte removal filter was equivalent to  $0.3 \pm 0.19\%$  gm /dl, in the order of 0.25% of the sample. Thus a minor degree of haemolysis appeared to be inevitable on preparing the erythrocytes for the filtration experiments.

These results suggest that the preparation of samples for erythrocyte filtration experiments may result in the loss and destruction of some erythrocytes. It cannot be assumed that the diabetic erythrocyte population is not altered by the erythrocyte preparation process. Potentially, the erythrocyte preparation process may remove subpopulations of cells, which would otherwise influence the filtration assay.

### 3-3-10 Destruction of Erythrocytes during Passage over the Nucleopore Filter

Some destruction of erythrocytes occurred on filtration of a  $0.1 \times 10^{12}/l$  concentration of erythrocytes over 3 micron nucleopore filters. There was visible haemolysis supernatant. (This was measured by spectrophotometry at a light emission of 415 nanometres as described above). The free haemoglobin in the part of the specimen passed over the nucleopore filter was equivalent to 0.073 gm/dl. There was no visible or spectrophotometric detection of haemolysis on filtration over the 5 micron size nucleopore filters. The 5 micron nucleopore was used for the erythrocyte filtration experiments.

### 3-3-11 A Comparison of the Efficiency of Cell Washing and Imugard Leucocyte Removal Filter in the Preparation of Leucocyte Free Erythrocyte Suspensions

In order to establish the most efficient method for the removal of leucocytes from the erythrocyte suspensions, a comparison was made of the efficiency of leucocyte removal by:

- 1) repeated cell washings (3 washings in PBS as described above)
- 2) pre-filtering the blood specimen over Imugard leucocyte removal filter (as described above)
- 3) a combination of 3 washings and pre-filtering over Imugard leucocyte removal filter.

Results are shown in Table 3-6. Almost all the platelets and 89% of the leucocytes (range 80.7 - 96.8%) were removed by 3 washes. After filtering over Imugard filter fewer platelets were removed, (the residual count was  $82.3 \pm 13.3$ ) and 71% of the leucocytes were removed, (range 59.2 - 90.8%). A combination of cell washings and pre-filtering over Imugard Leucocyte removal filter removed 93.6%, (range 90.8 - 96.4%) of the leucocytes and all the platelets. The combination of cell washing and pre-filtering was the most efficient method of removing leucocytes and platelets from the erythrocyte suspensions and was used in studies of normal and diabetic erythrocyte suspension filtration.

### 3-4 HAEMOSTATIC FUNCTION FACTORS

These were measured by the laboratory staff of the Department of Medicine in Leeds using standard assays. Blood for the assays of haemostatic factors was collected without stasis using a "Butterfly" cannula to allow rapid repeated sampling. Blood samples were collected into 120 mM trisodium citrate, 1 part citrate to 9 parts of blood unless otherwise stated. Two individuals were always involved in the collection of the blood samples; these were the author, who performed all the venesections and a member of the laboratory staff from the Department of Medicine at Leeds, who mixed the samples, placed them on melting ice, if appropriate, and arranged for immediate separation and storage of the plasma where necessary. Samples for the assay of Fibrinopeptide A were always collected first, followed by samples for other haemostatic factors, and then samples for blood chemistry and haematology. Before blood sampling, all volunteers and patients were required to lie quietly supine for 30 minutes.

#### 3-4-1 Factor VIII

Factor VIII has been studied extensively for a possible role in the development of vascular disease. It is a very complex molecule consisting of two components which, although linked, appear to be under separate genetic control (as reviewed by Hoyer, 1981). The first component, factor VIII:C (the factor VIII procoagulant protein or the antihaemophilic factor) is the procoagulant property of normal plasma that is measured in standard coagulation assays. Factor VIII procoagulant antigen levels are closely associated with assays of factor VIII:C activity. The site of synthesis of VIII:C is unknown but studies suggest it may be released by the liver (Owen et al 1979, Bloom 1979). It is generally agreed that VIII:C accelerates blood coagulation by its cofactor



role in the enzymatic activation of factor X by factor IXA and it is likely that thrombin activation is essential for VIII:C activity (Huftin and Nemerson 1978).

The bulk of the factor VIII complex consists of factor VIII-related protein, von Willebrand factor. Its structure and function have been reviewed by Hoyer (1981). Von Willebrand factor is a heterologous group of glycoproteins with a wide range of molecular weights. Von Willebrand factor is thought to bind to platelets and have a role in normal platelet aggregation and adhesion to damaged endothelium. The prolonged bleeding time in patients with Von Willebrand's disease is corrected by transfusion of Von Willebrand rich cryoprecipitate and there is a good correlation between this abnormality and that of ristocetin cofactor measurements of von Willebrand levels (Weiss et al, 1973). Thus ristocetin cofactor activity is essentially a measure of the biological activity of von Willebrand factor. However it is not known whether von Willebrand factor binds to platelets in the absence of ristocetin, nor how reduced level of factor VIII protein results in a prolonged bleeding time. Ristocetin cofactor activity does not always reflect the *in vivo* biological function of von Willebrand factor, as in some variants of von Willebrand's disease with near normal measurements of ristocetin cofactor activity, prolonged bleeding is still a problem (as reviewed by Hoyer, 1981). Thus, whilst it is believed that von Willebrand factor binding plays an important role in platelet aggregation and adhesion of platelets to damaged endothelium, there is no hard evidence.

Von Willebrand factor can also be measured by estimating antigen levels of factor VIII related protein, as distinct from measures of biological activity by ristocetin cofactor activity. A notable feature of factor VIII related protein is that it is partly synthesized by endothelial cells (Jaffe et al, 1973) and it has been suggested that the plasma level may be a useful marker for vascular endothelial damage (Boneu et al, 1975) even in early diabetic retinopathy (Porta et al, 1981)

#### a) Factor VIII:C

This was measured by a two-stage assay based on the thromboplastin generation technique. The basis of the technique is that clotting occurs when a substrate plasma is added to a pre-incubated mixture of Factor V, XII, XI, X and phospholipid (Factor VIII reagent, Diagen Kit) and Factor VIII supplied by the test plasma. The speed of clotting is determined by the amount of Factor VIII present in the test plasma. A simplified semi-automated version of the method was used (Denson & Wilkins, 1980). In this method, apart from the manual dilutions of test and standard plasmas, the procedure is automatic and more accurate than fully manual methods. The substrate plasma was treated with a resin and this dispenses with the need for additional calcium chloride in the second stage of the assay.

Blood for the assay was collected, without venous occlusion, into citrate. Doubling dilutions of the test plasma were made up in citrate saline, ranging from 1/16 to 1/256, and 0.05  $\mu$ litres of each dilution was added to each of two cuvettes of a Coag-a-Pet tray. A Coag-a-Pet Dual channel machine (General Diagnostics) was primed to deliver 0.2 ml of Factor VIII reagent. The test plasma and Factor VIII reagent mixture were incubated for 150 seconds (first stage) and then the machine delivered 0.1 ml of substrate plasma and the time to clot formation was recorded (second stage). A standard of fresh normal pooled plasma was run with each test plasma. The results of time to clot formation were plotted against the plasma dilution factor on log/log graph paper for both test and standard plasmas. The standard plasma was assigned a value of 100 percent and the test plasma value was extrapolated from the graph.

b) Factor VIII, von Willebrand related antigen (vWf:Ag)

This was measured by immunoelectrophoresis using the method of Laurell (1966). During electrophoresis of an antigen in an antibody-containing medium, antigen-antibody complexes will precipitate in a rocket-shaped zone. The height of the rocket will be proportional to the amount of antigen. In this assay agarose gel was used as the medium for containing the antibody to vWf:Ag. Pooled normal plasma was used as a standard. Dilutions of the standard and patient's plasma were prepared in 0.05 ml barbitone buffer and 5  $\mu$ litres of these were placed in small holes punched in the gel. After electrophoresis and staining with coomassie blue, 'rockets' of antigen-antibody complex appeared and their height measured. The standard curve was plotted on log/log paper and the concentration of vWf:Ag in the patient's plasma was extrapolated from this curve.

c) Ristocetin Co-factor

The basis of this assay is that ristocetin quantitatively agglutinates washed fixed human platelets in the presence of human Factor VIII, von Willebrand related protein. The method of Zuzel et al (1978) was used. Platelet-rich plasma was prepared and the platelet count adjusted to contain  $200-250 \times 10^9$  /litre platelets. A Payton Aggregometer was set for use at 37°C with a stirring speed of 900 rpm. A cuvette with platelet-rich plasma and a cuvette containing platelet poor-plasma were used to set the limits on the recorder scale. 0.5 ml of platelet rich plasma was placed in a cuvette, which was then warmed to 37°C. The cuvette was placed in the aggregometer and the ristocetin added. The platelet aggregation was recorded as a graphic representation of changes in the optical density of the cuvette. A standard reference curve was constructed by measuring the effect of different dilutions of pooled normal plasma on the aggregation of formalin-fixed platelets in the presence of ristocetin. The test plasma was then aggregated in duplicate in the presence of ristocetin and its percentage change in optical density noted. This was read off the standard curve and

expressed as a percentage of the activity of the normal pooled plasma (which was defined as 100%).

#### 3-4-2 Fibrinopeptide A (FPA)

Fibrinopeptide A is the 16-residue amino-terminal peptide that is cleaved from the A-alpha chain of fibrinogen by the action of thrombin, which is the initial step in the conversion of fibrinogen to fibrin (Nossel et al, 1971). Measurement of fibrinopeptide-A is thought to be a sensitive measure of in vivo thrombin activity (Nossel et al, 1974[a]). Less than perfect venesection technique results in spuriously high levels of fibrinopeptide-A presumably because the coagulation mechanism is activated at the site of venepuncture or within the needle and syringe. FPA was measured by radioimmunoassay using a commercial kit supplied by Imco (Sureden). The principle of the assay depends on separating the FPA by dialysis. It can then be quantified using 125-Iodine-labelled desaminotryrosyl-FPA with albumin-coated charcoal to separate the free and bound fractions.

Venous blood 4.5 ml was collected into a tube containing chilled anticoagulant (the dilution with blood forming final concentrations of EDTA 10m.mol/l, heparin 100 units/ml and trasylol 100 units/ml) and gently mixed and placed in a flask containing melting ice. Platelet-poor plasma was obtained by centrifugation at 2000g for 20 minutes at 4°C. The whole of the supernatant was removed and stored at -70°C until sufficient samples were available for assay by the kit.

#### 3-4-3 Fibrinogen

This was measured using the method of Ratnoff and Menzies, (1951). Fibrinogen is converted to a fibrin clot by the addition of thrombin to the test plasma. The fibrin clot is washed and treated with alkali. The amount of tyrosine present in the alkali-digested fibrin protein is read as a colour reaction in a spectrophotometer. A tyrosine standard is read at the same time as the test sample. The plasma fibrinogen is calculated by dividing the spectrophotometer reading of the sample by the spectrophotometer reading of the standard and this value is multiplied by a constant based on the standard amount of tyrosine in the fibrinogen/fibrin.

#### 3-4-4 Euglobulin Clot Lysis Time

Fibrinolytic activity in the author's studies was assessed by measurement of Euglobulin Clot Lysis Time, ECLT. This is a measure of plasminogen activators present in plasma and is based on the capacity of plasminogen activators to cause lysis of fibrin clots in the presence of plasminogen (Nilsson et al, 1978). The euglobulin clot lysis method is affected by both the fibrinogen and plasminogen content of the sample and the amount of inhibitor co-precipitated with the euglobulin fraction (Kluft, 1979). The ECLT

method has some limitations in the assessment of fibrinolytic capacity, which is generally equated with measurements of plasminogen activator. ECLT in effect measures the overall activity of the fibrinolytic plasminogen-plasmin system and apparent hypofibrinolysis cannot be attributed simply to a deficiency of plasminogen activator. Measurements of dilute euglobulin lysis times are not direct measurements of plasminogen activator nor can they be considered a complete assessment of fibrinolytic activity.

Dilute resting ECLT generally show a very wide range of resting values, and stimulation tests, provoking release of plasminogen activator, are thought to be a more accurate measure of the function of the fibrinolytic system. Release of plasminogen activator following venous occlusion forms the basis of the most commonly used test of fibrinolytic potential (Clarke et al 1960, Robertson et al 1972). Plasminogen activator is localized in the walls of small vessels and high levels of activity are also found in the vasa vasorum of the adventitia of large vessels (Todd, 1959). The media of large veins is moderately active and intimal cells only active when detached (as reviewed by Ogston, 1983 [c]). Besides venous occlusion pressure, other stimuli (including exercise, electric shock, nicotinic acid or des d-amino vasopressin (DDAVP) administration) also result in release of plasminogen activator by the vascular endothelium. The actual mediators of all these responses are unknown and their exact physiological significance is uncertain.

In the ECLT method, the test plasma is diluted and acidified to precipitate euglobulins. The euglobulin fraction contains most of the plasma fibrinogen and plasminogen, as well as the plasminogen activators and plasmin. The euglobulin precipitate is re-suspended in buffer, clotted by the addition of thrombin and incubated at 37 °C and the lysis of the euglobulin clot recorded. The lysis of the clot was measured in the Mallin automatic ECLT recorder. Tests were duplicated and the results expressed in minutes. Blood samples were collected without using venous occlusion after fasted subjects had rested supine for 30 minutes for measurements of resting basal ECLT, (R ECLT). Measurements of post-venous occlusion ECLT (PV ECLT) were taken from the opposite arm. Venous occlusion of 90 mm Hg was applied for 10 minutes. In some studies, measurements of ECLT were made 1 hour after subcutaneous injection of insulin (PI ECLT), and these samples were taken from a different vein where possible.

### 3-5 PERIPHERAL BLOOD FLOW AS MEASURED BY PLETHYSMOGRAPHY

#### 3-5-1 Introduction to the Principles of Plethysmography

Measurements of peripheral blood flow in the calf and forefoot were made by by the author, using an ECG triggered Periflow plethysmograph (Janssen Pharmaceutica, Janssen Scientific Instruments Division). In plethysmography the increase in volume of

a limb, which occurs with arterial input when venous output is occluded, is assumed to be a measure of the arterial inflow. The principle of plethysmography was conceived at the beginning of this century by Brodie (1905), as reviewed by Hyman and Windsor, (1961). Originally volume changes were measured by fluid displacement by the limb. Whitney introduced the strain gauge in 1951 and greater accuracy and semi-continuous measurement were made possible by the development of ECG triggering by Barendsen et al in 1971. The Periflow was developed in 1976 (Brugmans et al 1977).

### 3-5-2 The Periflow Plethysmograph

In the Periflow plethysmograph, the change in circumference of the limb on venous occlusion is measured by mercury filled silastic rubber gauges. The gauges are extremely light and stretchy and do not constrict the limb to any extent. Stretch of the gauge alters the electrical resistance of the fixed volume mercury column, as any change in length has to be accompanied by a corresponding change in area. The change in the electrical resistance of the mercury column is proportional to the change in volume of the limb and hence limb blood flow. A Bridge circuit converts the resistance variation into voltage variations and allows automatic zeroing of the mercury gauges. Thus measurements are independent of the initial strain or elongation of the mercury gauge. Good correlation has been obtained between distension of the gauge and limb volume (Englund et al, 1972) but the non-distensible part of the gauge, which measures 2.4 cm, does introduce error into the volume change measurements, greatest for small gauges. Venous occlusion is applied to the limb under study by automatic inflation of limb cuffs with air supplied from a compressed air source and a series of valves and reservoir drums which allow rapid cuff inflation and deflation. The casing for this part of the apparatus doubles as a table on top of which the patient lies for the examination. The fast acting pneumatic system of the Periflow allows rapid changes of venous occlusion and recovery periods which are timed by the ECG trigger or a manual programme.

### 3-5-3 Operation of the Periflow Plethysmograph

The Periflow pressure programme can be operated by either ECG triggering or by a manual programme with fixed rate occlusion at 3 or 60 beats per minute. The ECG triggering mechanism incorporates a variable delay. The arterial pulse takes longer to arrive at the feet than the forearm and the variable delay allows timing of cuff inflation to coincide with the arterial pulse for the limb under examination. The control panel contains separate venous and arterial occlusion programmes. The number of beats of venous occlusion and recovery can be varied from a range of 1 to 9 beats, but is generally set to supply venous occlusion for three beats and to deflate for two beats of the cardiac cycle. The venous occlusion pressure can be set from 0-99 mmHg but usually a pressure of 50 mmHg, which suppresses venous outflow but not arterial



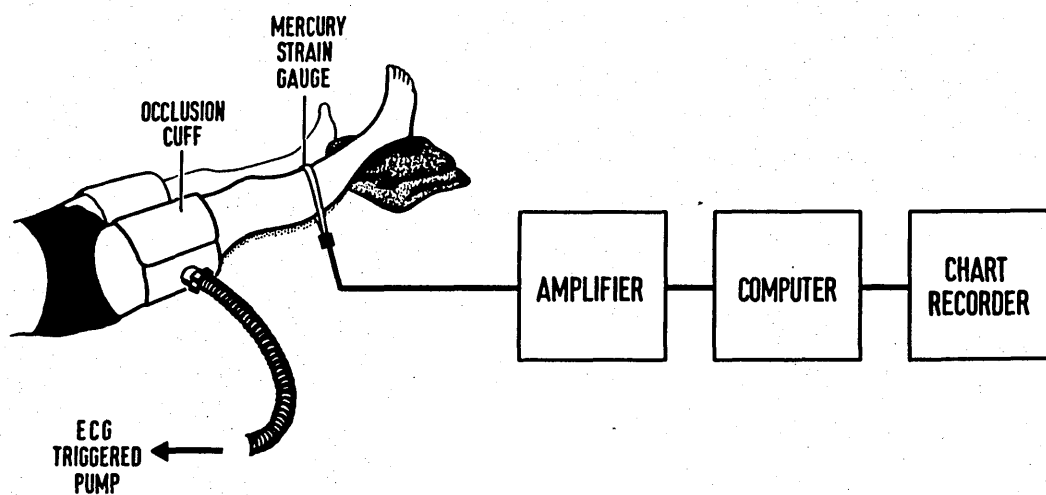
a) photograph of calf flow measurement



b) photograph of foot flow measurement

**FIGURE 3-6**

**The Set-up for Pleythsmographic Measurements of Peripheral Blood Flow**



c) diagram of apparatus

**FIGURE 3-6**

The Set-up for Plethysmographic Measurements of Peripheral Blood Flow

inflow, is selected. This has to be checked in individual patients. The arterial pressure programme used to apply arterial occlusion for measurements of reactive hyperaemia allows a wide range of occlusion pressures and times of occlusion. Generally a pressure at least 50 mmHg above the systolic pressure is chosen. After the variable time period for arterial occlusion the Periflow reverts automatically to the venous occlusion programme during which measurements of reactive hyperaemia are made. A re-set button allows instant cancellation of any programme. An integrated calculator converts the volume signal into a peripheral flow measurement taking into account settings on the occlusion programmes. Plethysmographic recordings of blood flow are usually recorded by chart recorder but may also be viewed by oscilloscope with addition of the relevant unit. The recorder selector module permits selection from several output signals including the Hg-gauge amplified output calibration signal, the differentiated input signal of the Hg-gauge amplifiers, the calculated volume changes by venous occlusion-calibration, the calculated volume changes of initial volume after arterial occlusion, a QRS monitoring signal, the triggering delay of the ECG, and the cuff pressure calibration. Photographs of a subject positioned for plethysmographic measurements of blood flow of (a) the calf and (b) the foot are shown in figure 3-6 and schematically represented in figure 3-6 (c).

#### 3-5-4 Protocol for Plethysmographic Measurements of Blood Flow.

Blood flow in the peripheries is affected by temperature (Freeman, 1935; Felder et al, 1954) and limb position in relation to the heart is also important (Barendsen et al, 1971). In order to introduce some uniformity blood flow measurements of diabetic patients were always performed in the morning, after breakfast and the patients were allowed to acclimatize for 30 minutes at  $24.5 \pm 0.5^{\circ}\text{C}$  in the constant temperature room. Skin foot temperature was measured by a thermocouple attached to the skin of the dorsum of the foot below the web between the first and second toes. In individual patients the same strain gauge was used to record flow on each occasion and was placed in the same position for each recording. Measurements were made of resting calf blood flow and resting forefoot blood flow and reactive hyperaemia of the forefoot after a 4 minute period of arterial occlusion. The calf gauge was placed round the broadest part of the calf muscle, and a note was made of the distance from the lower patella border to allow accurate placement in repeat studies. The foot gauge was placed round the broadest part of the foot, measured down from the medial malleolus and was fastened over the bony dorsum of the foot. The patients were assessed in the supine position with the shoulders on the bed and head on a double pillow. The legs were elevated at the feet by 15 cm and allowed to rest held straight in slight external rotation. ECG leads were attached and the plethysmograph was operated by ECG triggering. The cuffs of the plethysmograph were attached round the thighs for assessment of calf flow and just above the ankles for assessment of foot flow. The



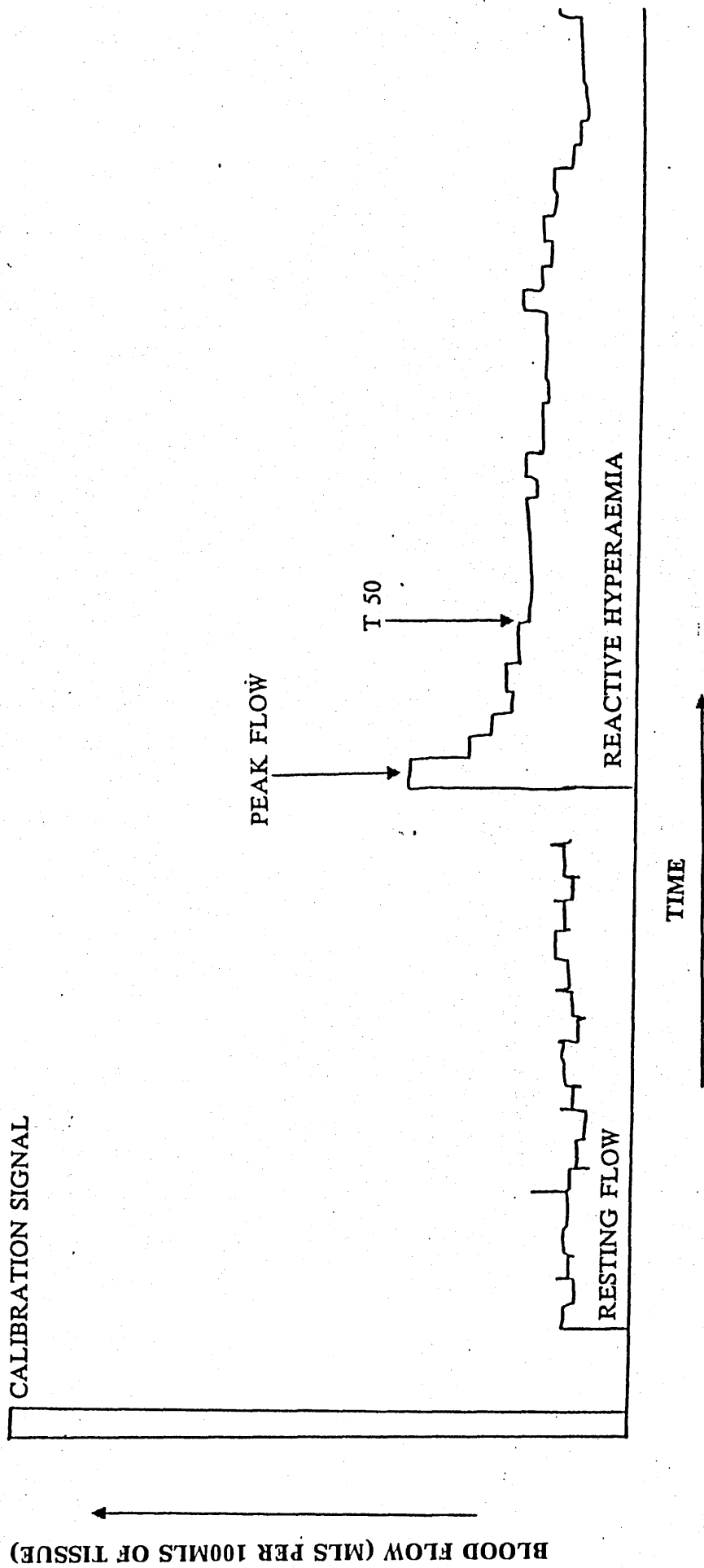


FIGURE 3-7

Examples of Periflow Plethysmograph Recordings of Blood Flow

measurements of flow were generally made in the right leg but if varicosities or some other abnormality was present (e.g. previous injury) measurements of the left leg were made. The plethysmograph was programmed to inflate for 3 beats of the cardiac cycle and to deflate for 2 beats of the cardiac cycle. The venous occlusion pressure was 50 mmHg. Resting calf flow was recorded over a period of 3 minutes. After calf flow measurements, the thigh cuffs were removed and the ankle cuffs and gauges positioned for the forefoot measurements. Resting forefoot flow was measured over a 3 minute period. The reactive hyperaemia following a 4 minute period of arterial occlusion was then recorded over a 3 minute period. The arterial occlusion pressure was 250 mmHg. The recorder selector programme was set to the output from the mercury strain gauge and to the computerized flow values on the second channel. The results were recorded on a two-pen graph recorder, and before commencing measurements calibration signals for the computerized flow value - equivalent to 30 ml blood per 100 ml tissue and for the mercury strain gauge output 0.1, 0.5 and 1% volume change were obtained. Examples of the blood flow recording graph are shown in Figure 3-7.

Resting flow values of the calf, Resting Foot Flow (RCF) and foot, Resting Foot Flow (RFF) were calculated by using a planimeter to measure the area under the graph of the computerized flow output signal. Flow values per 100 ml tissue per minute were calculated by taking a ratio of the area under the rest flow area to the area under the calibration signal. Reactive hyperaemia was assessed by measuring the time to each peak flow values, (TPF) the peak flow value following arterial occlusion (PF) and peak flow minus resting flow (PF-RF) and the time for the peak flow value to reach half its value, T50, see figure 3-7. For some of the flow recordings the arterial debt was calculated by multiplying rest flow by the time of arterial occlusion. The rate of pay off of the arterial debt was then calculated by looking at the percentage paid back in 0- 30 seconds.

### 3-6 LASER DOPPLER FLOWMETER MEASUREMENTS OF SKIN BLOOD FLOW IN THE PAD OF THE GREAT TOE

#### 3-6-1 Introduction and Principles of the Laser Doppler Flowmeter

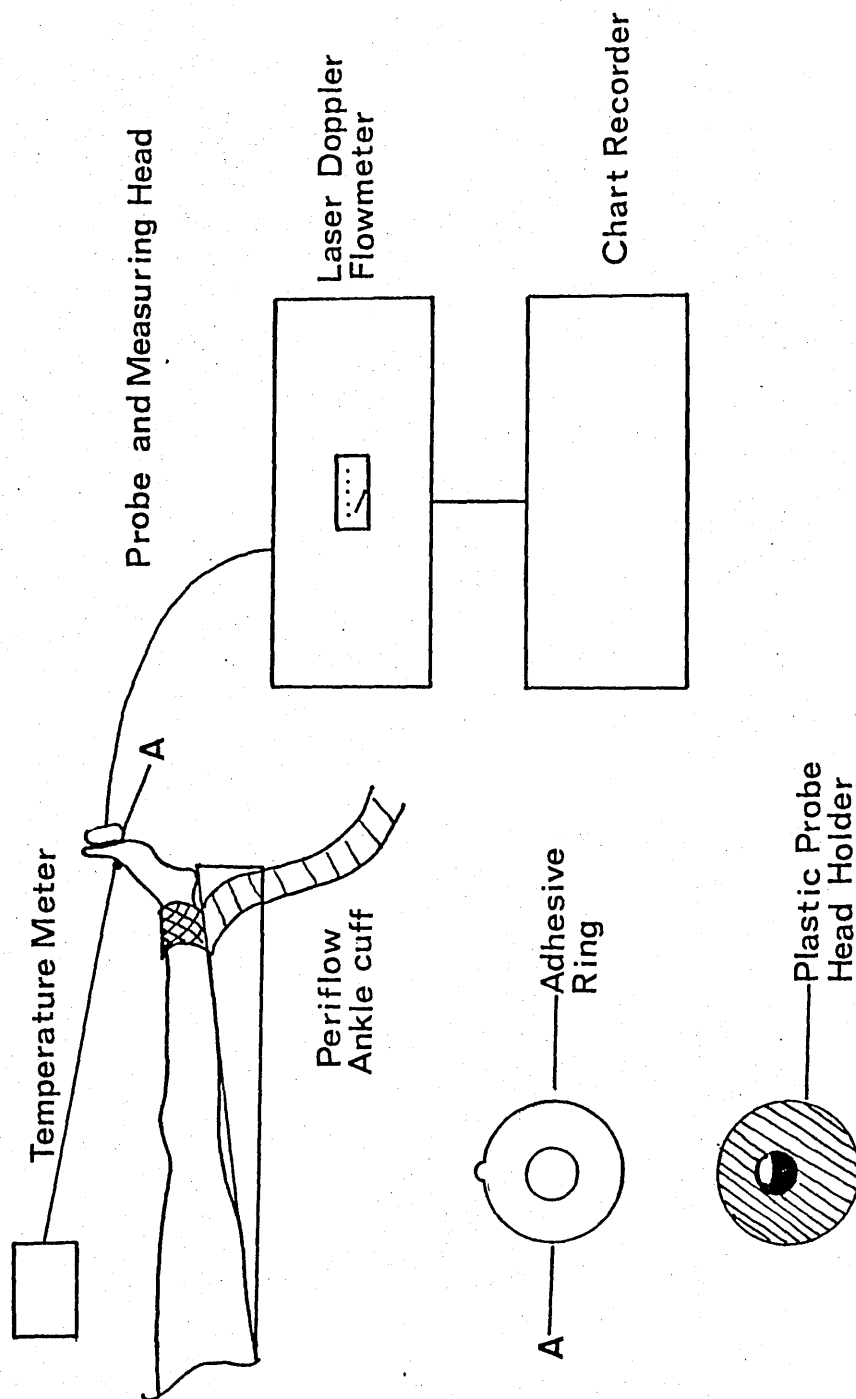
Blood flow at the great toe pad was measured by the author with a Periflux Laser Doppler Flowmeter, which is a helium neon laser. The principle of the laser doppler flowmeter is that a laser light beam incidence on tissue is scattered both on static structures and on moving structures, i.e. red cells. Light beams scattered on moving matter undergo a frequency shift according to the Doppler effect, whilst beams scattered in static tissue alone maintain the same frequency. A portion of the back scattered light is brought to impinge on the surface of a photodetector where beat notes produced by mixing of waves scattered in different structures are formed. From the

alternating portion of the photocurrent, an output signal that is related to the flux of red cells in a specific tissue volume is generated (Tenland, 1982).

The laser doppler measures movement within a hemisphere volume some 1-1.5 mm from the laser's circular measuring head front surface. The signal records movements mostly of erythrocytes but lesser contributions are received from all other cellular components and from vessel walls and other membranes. The perfused flowmeter does not react to the flow of fluids, such as plasma, which have a uniform refractive index. The signal obtained is independent of direction of structure movement but in vitro correlates positively with the number of blood cells in motion and their velocities (Nilsson et al, 1980). Movement of the measuring head towards or away from the tissue to be studied also gives rise to a signal. The primary signal is converted into an analogue voltage and displayed, graduated in percent on an analogue meter. A chart recorder is attached to the recorder output to obtain a permanent record of blood flow recordings. The reading and output signals are proportional to blood cell flux and is a relative rather than absolute value.

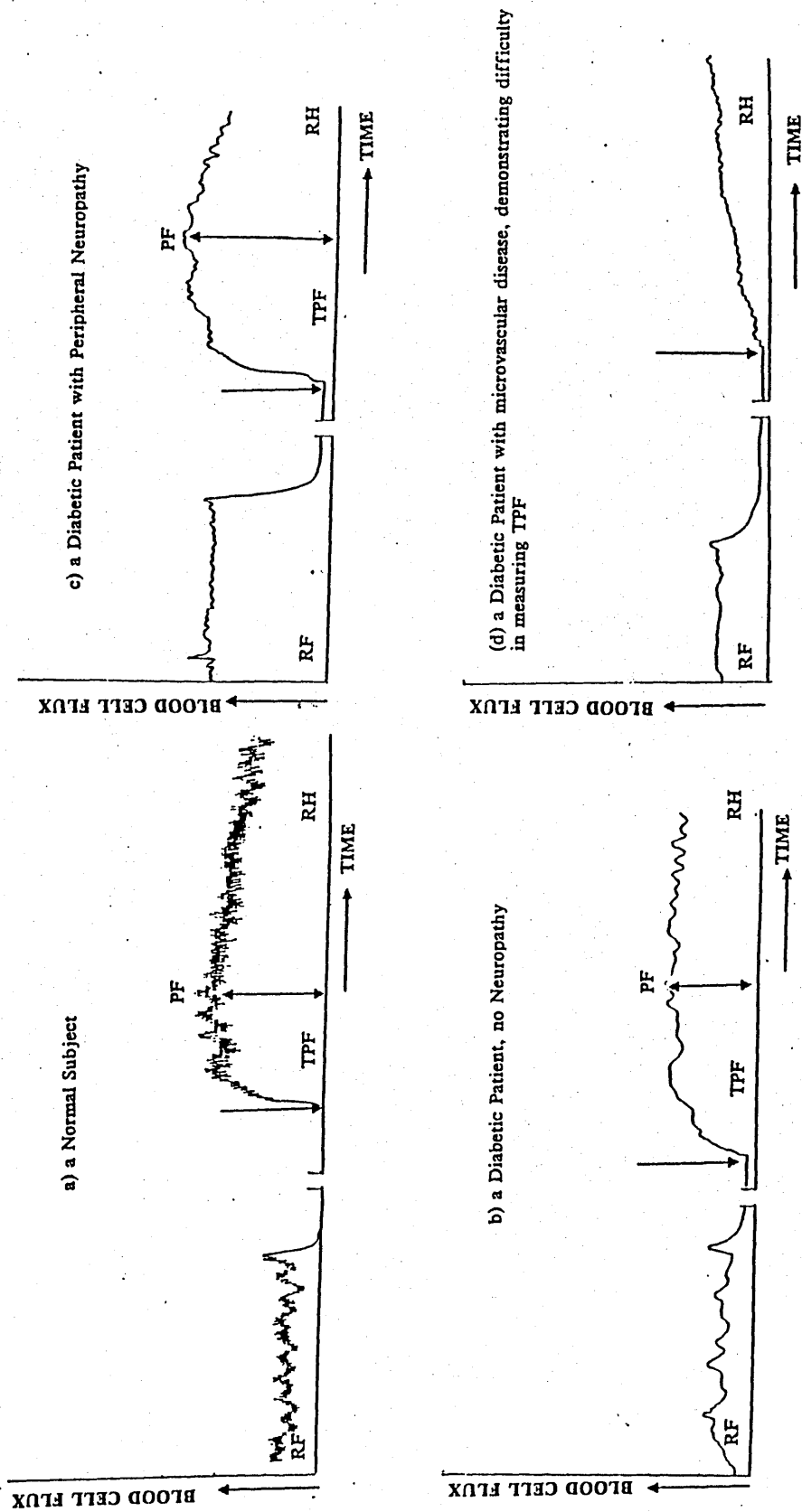
### 3-6-2 Factors Influencing Laser Doppler Measurements of Blood Flow.

The use of the laser doppler flowmeter for measuring skin blood flow was suggested by Stern in 1975. The thickness of the overlying keratin layer will affect the depth of dermal penetration by the laser beam and hence the vessel in which blood movement is recorded. In the skin of the fingers a 200-400  $\mu\text{m}$  keratin layer separate the capillaries which measure 0.2-0.4 mm from the laser doppler. Thus theoretically blood flow measurements will be predominantly of capillary flow but flow in deeper vessels may well be recorded. Skin blood flow is extremely variable from site to site and in individuals day to day variations are very great making it impracticable to define normal flow levels (Tenland, 1982). Rhythmical variations in human skin blood flow occur which are non-synchronous with respirations and heart rate and these too vary between different sites and individuals. These are thought to be due to spontaneous rhythmical contractions of smooth muscles surrounding the small vessels (Salerud et al, 1983). The effects of various standard stimuli such as ischaemia and deep inspiration on skin blood flow can be demonstrated by measurements with the laser doppler flowmeter. The advantages of the laser doppler flowmeter are that it does not interfere with the tissue under study but disadvantages include sensitivity to movement artifact, the unknown relationship of measurements with tissue pigmentation, epidermal thickness and blood haemoglobin and the poor reproducibility and enormous variations within individuals which make repeated follow-up studies difficult and establishing a normal range difficult.



**FIGURE 3-8**

Diagram of Arrangements for Laser Doppler Flowmeter Recordings of Toe Pad Flow



**FIGURE 3-9**

Examples of Toe Pad Flow Recording by Laser Doppler Flowmeter

### 3-6-3 Protocol for the Measurement of Toe Pad Blood Flow

Toe pad blood flow measurements were performed by the author in the constant temperature room at an ambient temperature of 24°C. The set-up for the laser doppler flow measurements is represented schematically in Figure 3-8. Subjects were acclimatized for approximately forty-five minutes before measurements were made (which followed directly on from the plethysmographic recordings of resting calf flow). The subject was positioned as for plethysmographic blood flow measurements, supine with the leg elevated by approximately 15 cm and supported at the ankle.

Automatically inflating ECG triggered pressure cuffs from the periflow plethysmograph were attached around the ankle. The laser doppler probe was attached to the great toe pad for measurements by means of a double-sided adhesive ring and plastic probe head holder. Temperature was recorded from the web between the great and second toes. The laser doppler flow meter was allowed to warm up for 15 minutes before use. The record output channel was attached to a graph recorder, paper speed 50 mm/minute, and the 100% signal was calibrated to 10 volts. The periflux zero was obtained by first attaching the probe head to the solid white front of the console panel of the laser doppler panel. The gain was set at X3 and the frequency limit at 4 kHz, output circuit time constant 3.0 seconds.

Measurements were made of resting toe pad flow and reactive hyperaemia during plethysmographic recordings of resting foot flow and reactive hyperaemia following arterial occlusion at the ankle for 4 minutes. Movements of the plethysmograph gave rise to considerable artifact therefore the only feature of toe blood flow measured was time taken to reach peak flow values following release of the arterial tourniquet. Peak flow was defined as the maximum deflection following release of the tourniquet. Examples of Laser Doppler Flowmeter recordings are shown in Figure 3-9.

### 3-7 ELECTROPHYSIOLOGICAL STUDIES OF NERVE FUNCTION

The electrophysiological studies were made by Dr A Twomey of the Department of Neurophysiology in Leeds General Infirmary. Electrophysiological studies consisted of motor conduction velocities (MCV) and F response latencies of the Ulnar and Common Peroneal nerves, and sensory conduction velocities (SCV) of the Ulnar and Sural nerves using the methods described in Lenman and Ritchie (1983). Motor nerves were stimulated maximally with fixed-duration square wave pulses and the responses recorded using surface electrodes. Sensory nerve action potentials were recorded antidromically in the hand (using stimulating ring electrodes) and orthodromically in the legs. The F response latency was measured as the fastest latency in a group of 20 responses. All electrophysiological measurements were performed in a well heated room using a Medelin MS6 Recorder. Surface temperature was recorded at each visit using an Edale digit 1 thermometer.

### 3-8 ASSESSMENT OF DIABETIC EYE DISEASE

Assessment of diabetic eye disease was performed by members of staff of the Department of Ophthalmology at Leeds General Infirmary. Visual acuity was measured and the eyes were examined by an ophthalmologist (after dilation of the pupils using a short acting mydriatic), both by slit lamp and by ophthalmoscope. A note was made of the presence or absence of cataract, haemorrhages, hard exudates, cotton wool spots, microaneurysms, new vessel formation and other retinal abnormalities.

Colour slide photographs and fluorescein angiograms of the retinae were taken at the beginning and at the end of the trial to provide a record of progression of microvascular disease. The eyes were fully dilated as before and the plain photographs were taken with a Canon Fundus camera model CF-60Z using a 60 degree field of view centre on the macula. An injection of 5 ml of 20% fluorescein (SAS Pharmaceutical, Wycombe End, Beaconsfield, Bucks) was given intravenously by rapid bolus injection by the author. Photographs (black and white) were taken commencing seven seconds after the injection at a rate of one frame a second for twenty seconds. The right eye was usually photographed first and then a few late shots of the left eye were taken. Residual shots were taken of both eyes five minutes after the fluorescein injection.

The colour slides and fluorescein angiograms of the retinae were assessed by a second ophthalmologist. Assessments were made by viewing the negatives through a magnifier and counting the number of lesions in the whole field. A record was made by simple counting of the presence or absence and severity of the following lesions:

- 1) microaneurysms
- 2) haemorrhages
- 3) hard exudates
- 4) cotton wool spots
- 5) areas of capillary non-perfusion
- 6) new vessels
- 7) areas of laser treatment.

The number of microaneurysms was classified as

- + = sparse lesions <5;
- ++ = moderate number of lesions <20;
- +++ = numerous lesions >20.

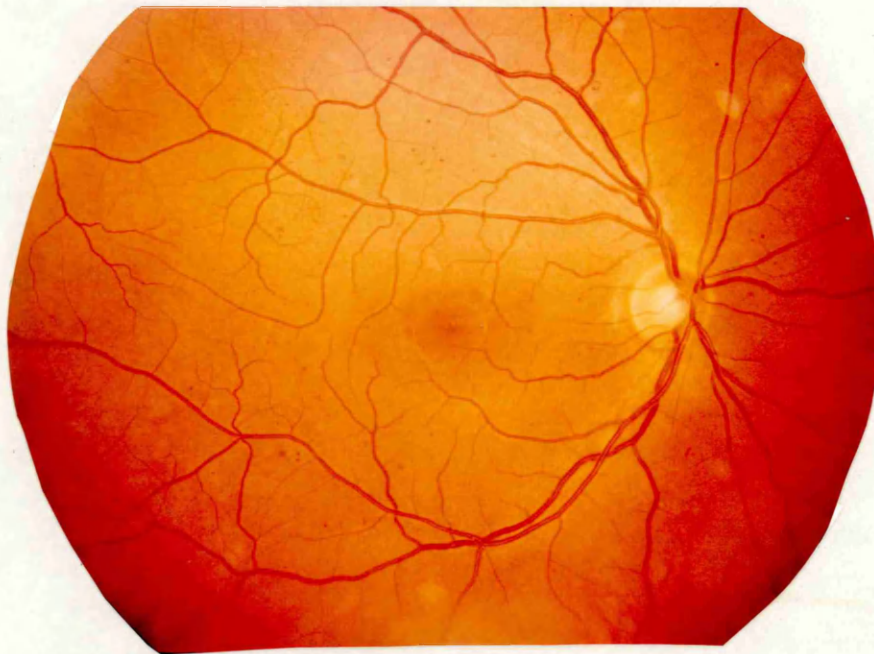
Each eye was classified as having either background or proliferative retinopathy (Benson et al, 1981) and a note was made of advanced background disease considered

to be pre-proliferative on the basis of areas of capillary non-perfusion, presence of large blot haemorrhages and multiple cotton wool spots (Kohner, 1976). The presence of maculopathy was also recorded. A comparison of the state of the eyes at recruitment to the trial and at the end was made on the basis of changes in the plain fundal photographs, fluorescein angiograms and clinical events by the second ophthalmologist and the author. Examples of the fluorescein angiograms and plain fundal photographs are shown in Figures 3-10, 3-11 and 3-12.

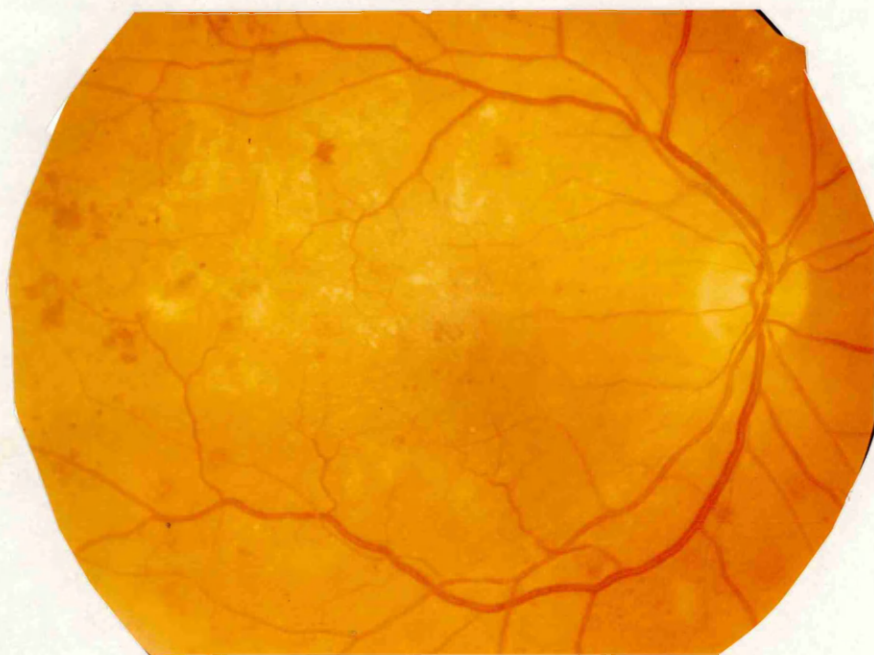
### 3-9 STATISTICAL ANALYSIS

Details of the statistical tests used are given separately for each results section. As biological data cannot be assumed to be normally distributed, non-parametric statistical tests were often employed in order to avoid errors due to skewed data. Where appropriate parametric statistical tests were also used. The analysis was made using a statistical package, OXSTAT: except where statistics for small numbers were performed manually. The multiple and stepwise linear regression analyses were performed using the statistical package MINITAB.





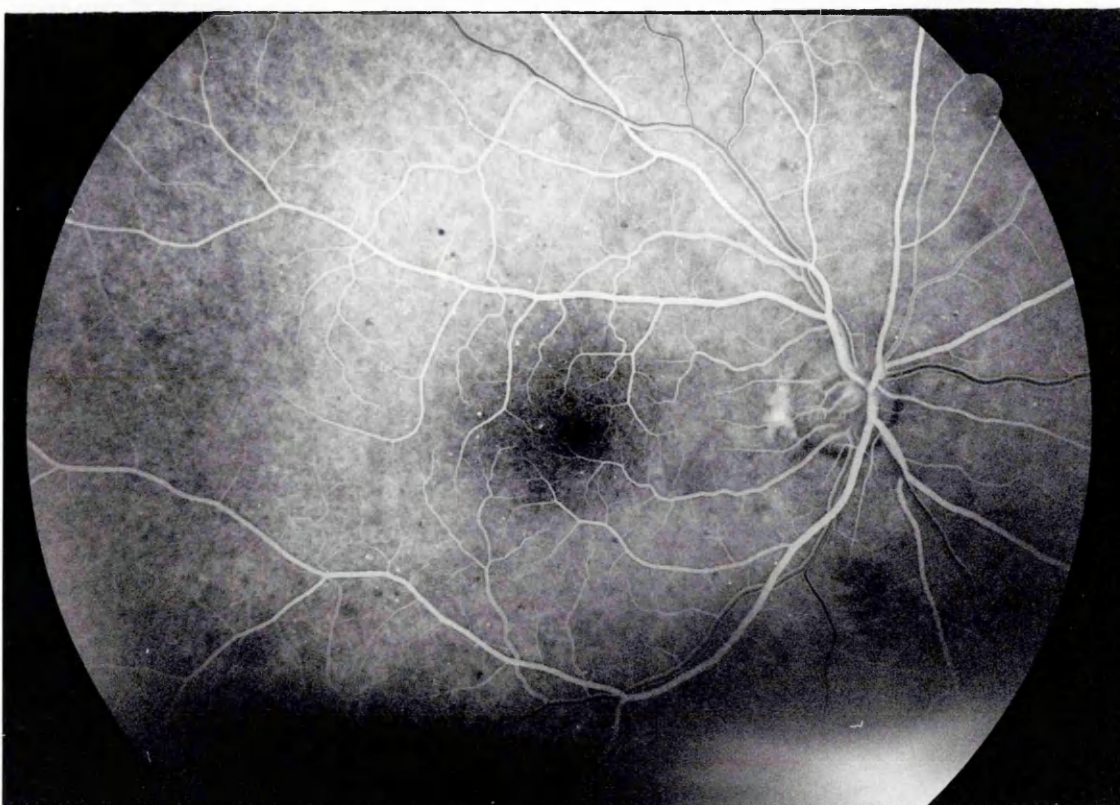
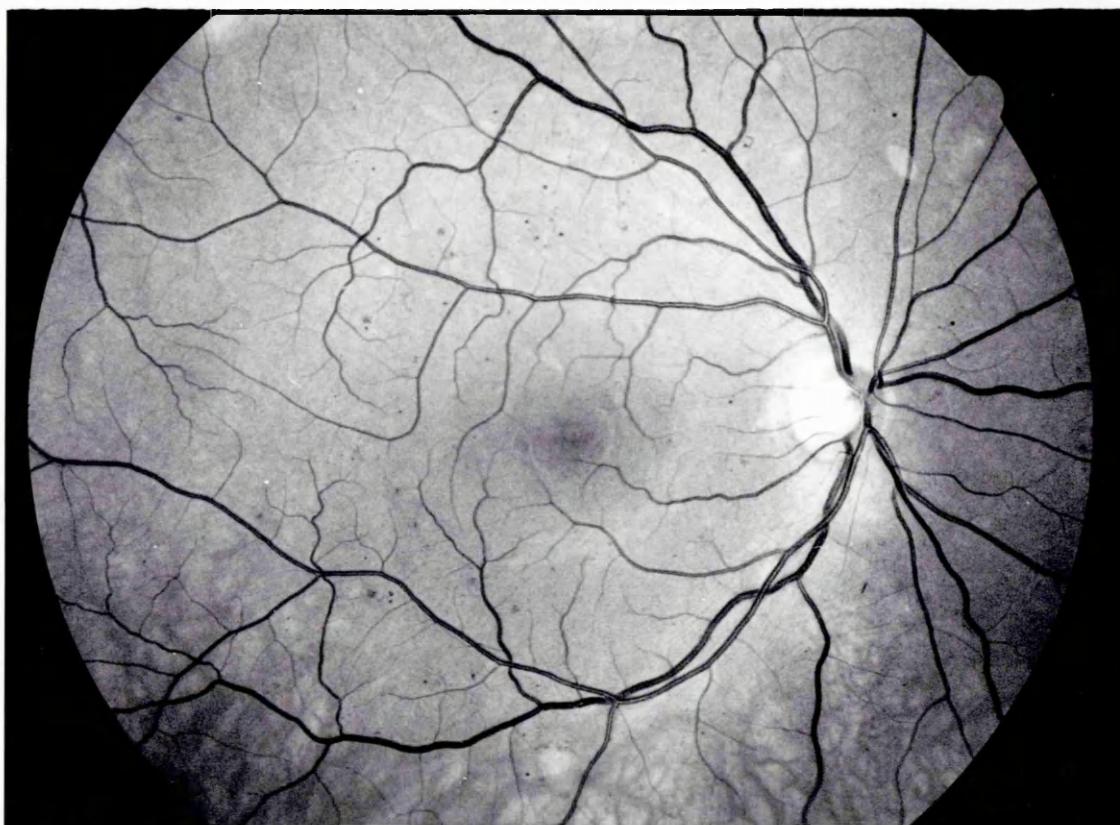
a) RG, background retinopathy



b) RoK, advanced background retinopathy

**FIGURE 3-10**

**Plain Fundal Photographs of Diabetic Retinae**

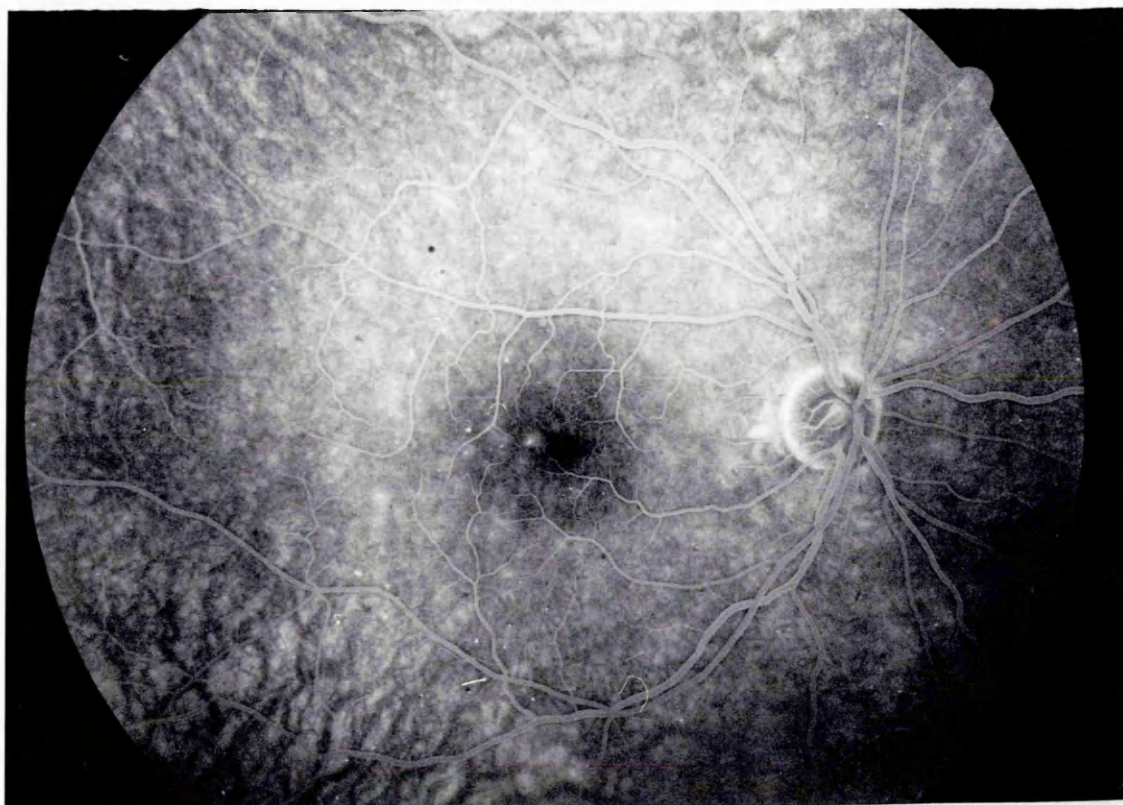
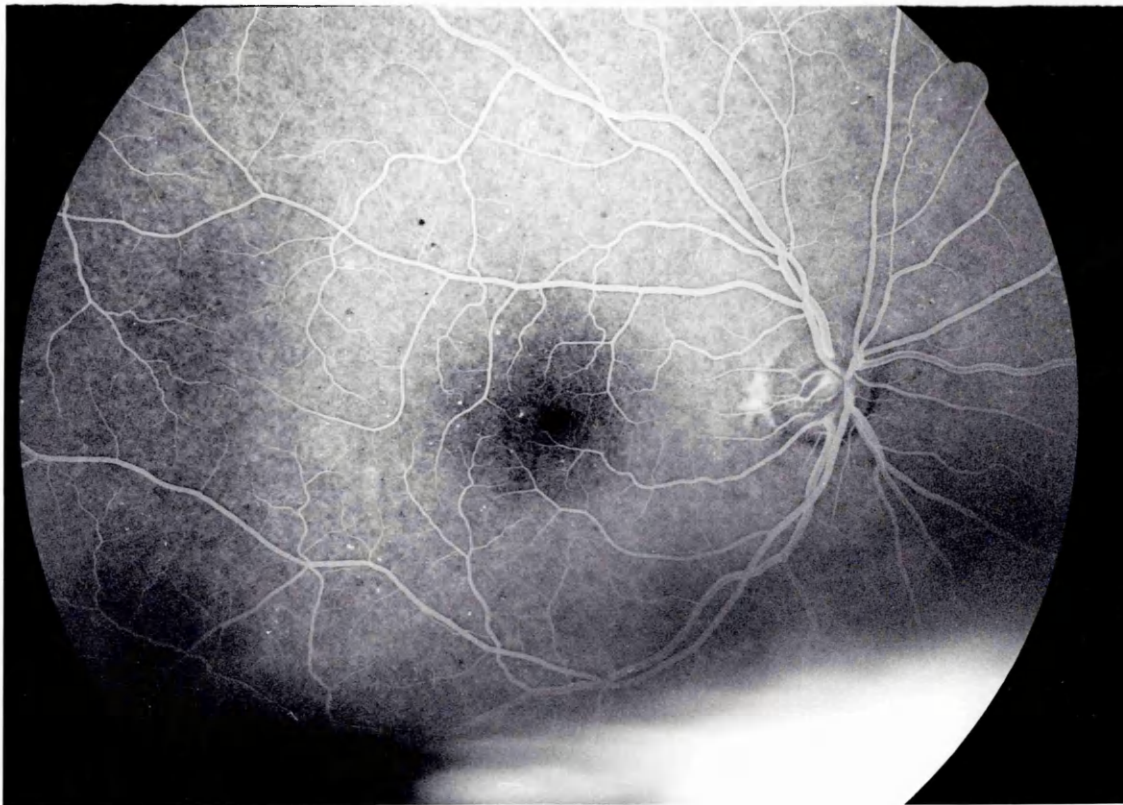


**FIGURE 3-11**

**Fluorescein Retinal Angiogram of Background Diabetic Retinopathy**

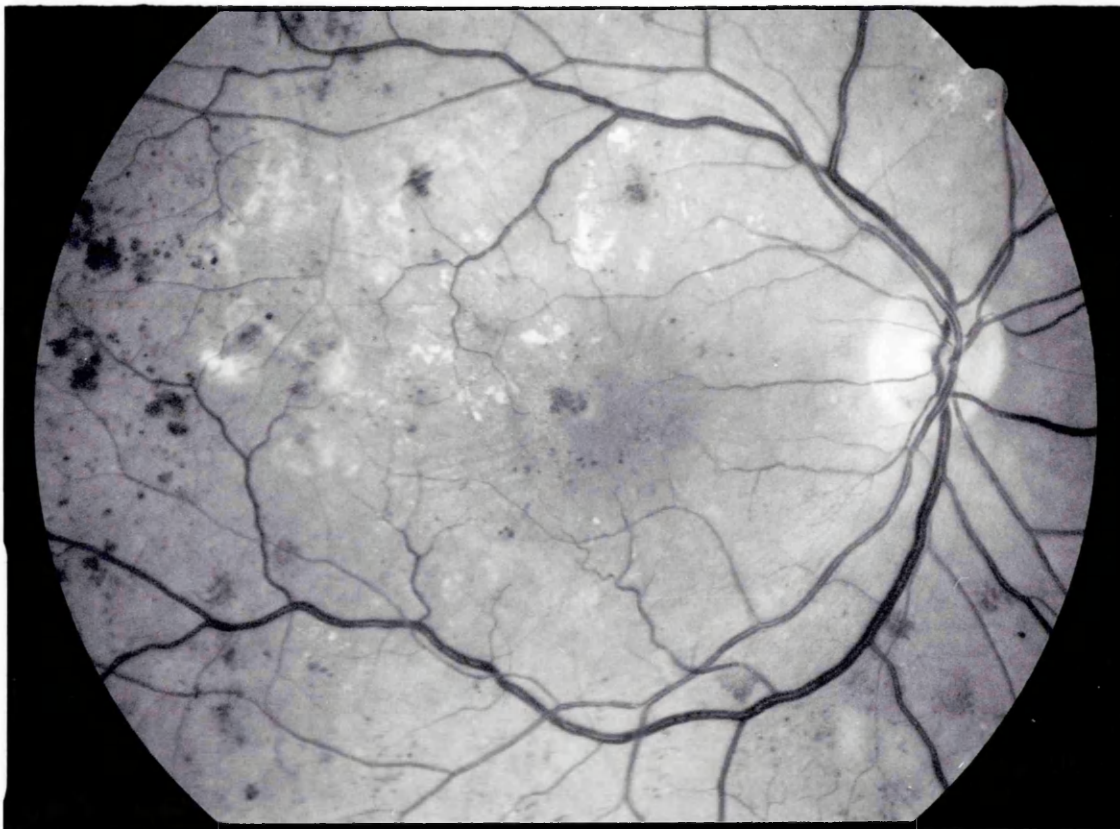
**a) early sequence**





**FIGURE 3-11**

**Fluorescein Retinal Angiogram of Background Diabetic Retinopathy**  
**b) late sequence**

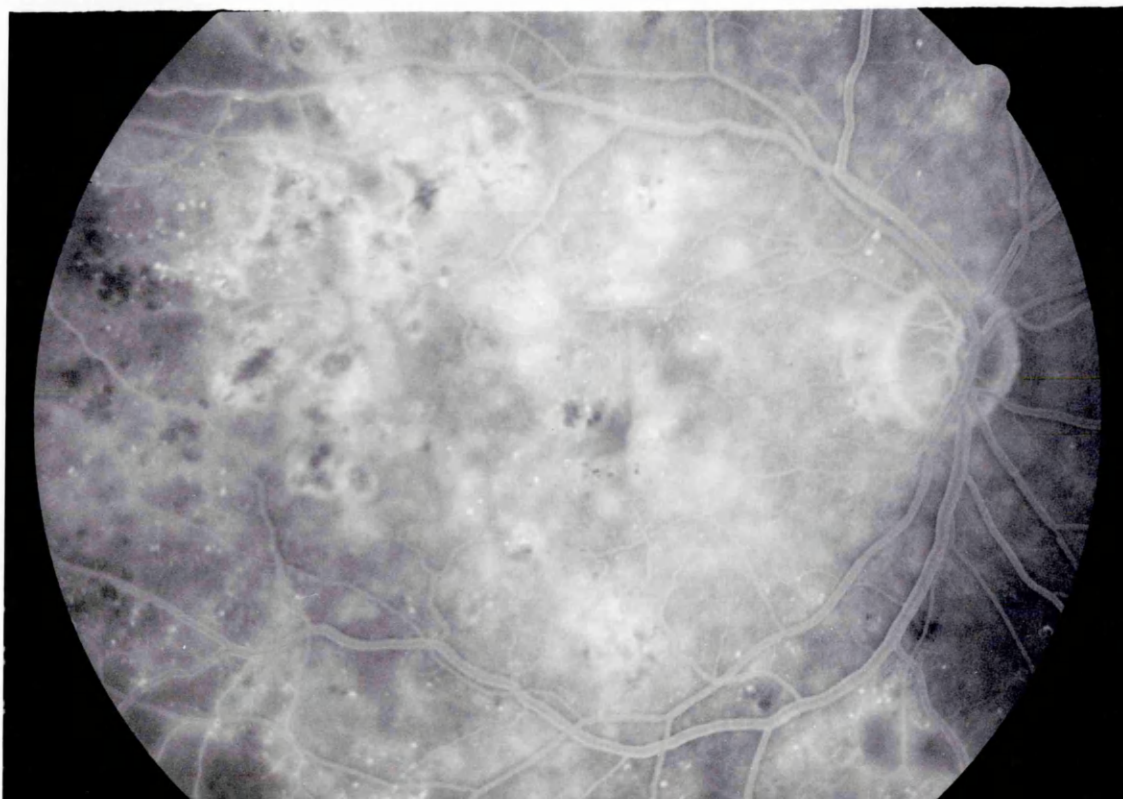
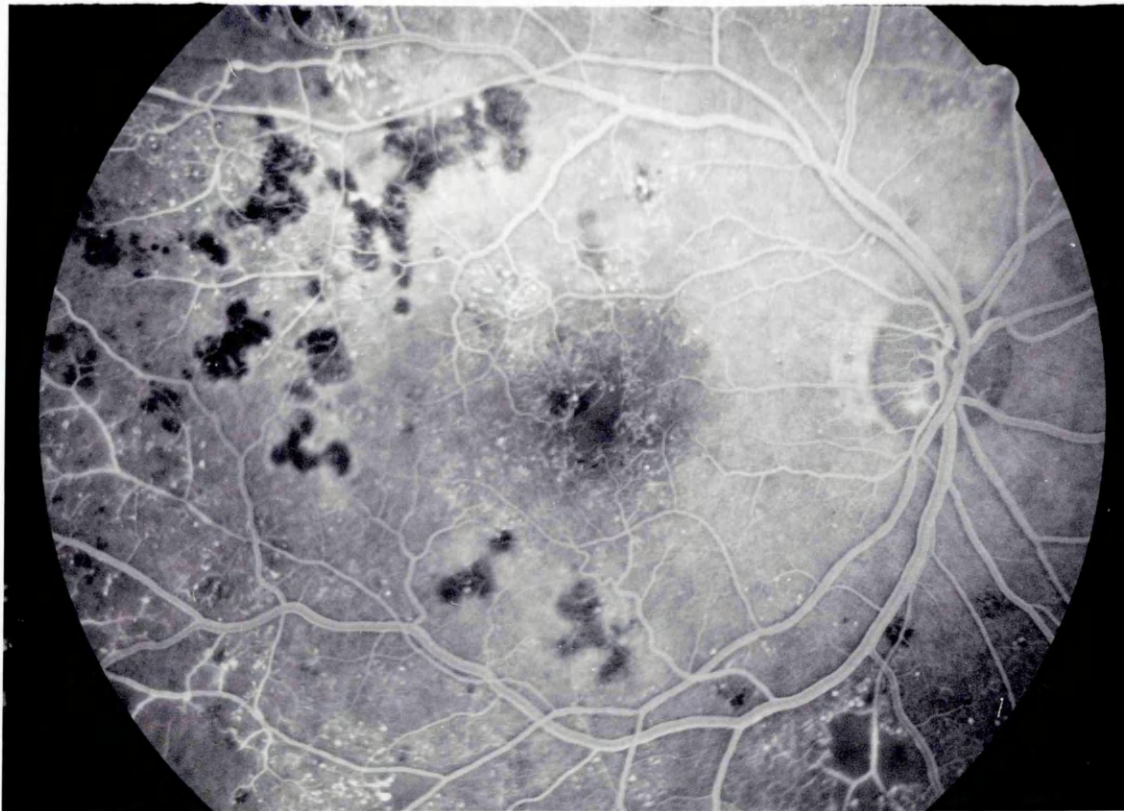


**FIGURE 3-12**

**Fluorescein Retinal Angiogram of Advanced Background Retinopathy**

**a) early sequence**





**FIGURE 3-12**

**Fluorescein Retinal Angiogram of Advanced Background Retinopathy**

**b) late sequence**

**APPENDIX**

**TABLES TO CHAPTER 3**

TABLE 3 - 1

## Reproducibility of Whole Blood Viscosity Measurements

Shear Rates (secs <sup>-1</sup> )	Graph Reading	Mean	C.V.	Mean whole blood viscosity (centipoise)		Conversion factor (Reading to Centipoise)
2.62	42	42.6	3.2%	12.8		0.301
	42					
	42					
	41					
	40.5					
	44.5					
	44					
	43					
Sample 1 N = 10	41.5					
	44					
0.130	60	59.7	4.2%	72.69		1.22
Sample 2 N = 3	57					
	62					
0.130	45	46.5	11.2%	56.7		1.22
	40					
Sample 3 N = 4	52					
	49					
0.130	53	52.4	6.8%	63.9		1.22
	55					
	56					
Sample 4 N = 5	47					
	51					
0.130	46	46.5	5.7%	56.8		1.22
	48					
Sample 5 N = 4	43					
	49					

Table 3 - 2A and 3 - 2B

## Reproducibility of Erythrocyte Suspension Filtration

## 3 - 2A Intra-Assay Co-efficient of Variation

Suspension WCC $10^7/L$	Suspension RBC $10^{12}/L$	Filterability mmHg	mean mmHg	SD	CV%
2.2	1.0	2.4	2.48	0.26	10.5%
2.2	1.0	2.3			
2.2	1.0	2.4			
2.2	1.0	2.6			
2.2	1.0	2.5			
2.2	1.0	2.2			
2.2	1.0	3.0			

## 3 - 2B Inter-Assay Variation

Specimen	Suspension WCC $10^7/L$	Suspension RBC $10^{12}/L$	Filterability mmHg
A Day 1	2.2	1.0	2.5
Day 3	1.5	1.0	1.1
Day 5	2.7	1.0	3.5
B Day 1	0.8	2.0	1.0
Day 3	3.15	2.0	1.2
Day 5	9.0	2.0	9.0

Suspension WCC - the calculated suspension leucocyte concentration  $10^7/L$  obtained from multiplying the leucocyte count of washed and filtered cell preparations by the dilutions factor used to make up  $1.0 = 10^{12}/L$  RBC suspensions.

Suspension RBC - erythrocyte count  $10^{12}/L$ .



Table 3-3

Changes in Intracellular Ion Content of Erythrocytes  
prepared for Cell Filtration

Sample		Intracellular sodium m.mol/L	Intracellular potassium m.mol/L
Whole Blood	1	11.45	82.78
	2	11.32	79.90
	$\bar{X}$	11.38	81.34
Washed Cells	1	11.36	80.30
	2	12.11	74.50
	$\bar{X}$	11.73	77.4
Filtered Cells 1		11.33	82.32
Washed and Filtered Cells	1	11.38	77.64
	2	12.11	76.22
	$\bar{X}$	11.74	76.93

\* Whole blood for samples 1 and 2 was collected from the same individual on 2 separate occasions.

Table 3 - 4

The Effects of Varying Glucose Concentrations of the Suspending Buffer on Erythrocyte Suspension Filterability mean (  $\pm$  SD)

Buffer Glucose concentration mmol/L	0	5	10	20	30
Filterability mmHg N = 9	1.8 ( $\pm$ 1.0)	2.1 ( $\pm$ 1.1)			
Filterability mmHg N = 6		3.9 ( $\pm$ 3.4)	3.1 ( $\pm$ 3.0)	3.1 ( $\pm$ 2.9)	2.6 ( $\pm$ 2.2)
Filterability mmHg N = 12		2. ( $\pm$ 2.3)		2.9 ( $\pm$ 2.8)	
Filterability mmHg N = 10		2.3 ( $\pm$ 3.0)			2.1 ( $\pm$ 1.8)

Table 3 - 5

Changes in Erythrocyte M.C.V. during Preparations  
of Erythrocyte Suspensions

Blood Samples	M.C.V. mean $\pm$ SD			
	Whole Blood	Washed RBC	Filtered RBC	Washed and Filtered RBC
N = 8	88.1 $\pm$ 4.2	87.9 $\pm$ 4.4	87.6 $\pm$ 4.5	
N = 13	90.5 $\pm$ 5.3			90.8 $\pm$ 5.2
Microcytic Blood Sample N = 1	62.2	68.0	68.0	
Macrocytic Blood Sample N = 1	105.5			105.8

Table 3 - 6

A comparison of the Efficiency of Leucocyte Removal from Erythrocyte Suspensions by Washing, Pre-Filtering over Leucocyte Removal Filter and by a Combination of the two processes (means  $\pm$  S.D.)

N = 3	Whole Blood	Washed	Pre-filtered	Washed and Pre-filtered
% Removal of Leucocytes	0	89.0 $\pm$ 8.1	71.0 $\pm$ 17.2	93.6 $\pm$ 2.8
Platelet Count $10^9$ /L	346 $\pm$ 32.9	2.3 $\pm$ 1.5	82.3 $\pm$ 13.3	2.7 $\pm$ 1.1
M C V	86.0 $\pm$ 5.0	85.9 $\pm$ 5.1	86.7 $\pm$ 5.1	86.2 $\pm$ 4.9

## CHAPTER 4

### ERYTHROCYTE FILTERABILITY RESULTS

#### 4-1 INTRODUCTION

The studies of erythrocyte filterability presented in this thesis were commenced at a time of growing controversy concerning the authenticity of filtration techniques as a measure of erythrocyte deformability. Plasma proteins, leucocytes, platelets and erythrocyte aggregation had been shown to influence measurements of erythrocyte deformability made by whole blood and concentrated erythrocyte suspension filtration (as reviewed by Boisseau, 1981), and much of the evidence for reduced erythrocyte deformability in diabetes mellitus has been based on studies using these techniques (Schmid-Schonbein & Volger, 1976; Barnes et al, 1977; Juhan et al, 1978). In diabetes mellitus, plasma fibrinogen levels are commonly elevated (see chapter 1, section 1-6) and poorly controlled diabetic patients may have high leucocyte counts. These observations suggest that the currently held hypothesis, that microvascular damage in diabetes is partly a consequence of i) restricted entry of less deformable erythrocytes into small blood vessels, so leading to impaired tissue nutrition and ii) vessel wall damage consequent on shear stress injury caused by hardened erythrocytes (reviewed by McMillan, 1983), might be invalid.

However, Juhan et al, (1982) reported that insulin, either in vivo or in vitro, could reverse the impaired filtration of diabetic erythrocytes in 40% suspensions. This effect was apparently a consequence of a direct insulin effect on erythrocyte membrane (Bryszewska & Leyko, 1983). These findings apparently confirmed the established hypothesis. In order to resolve these issues, the following studies were undertaken:

- 1) the effect of leucocyte contamination on erythrocyte suspension filtration was measured
- 2) the effects of insulin on erythrocyte suspension filterability were determined
- 3) the filterability of erythrocyte suspensions prepared from blood of normal and diabetic subjects was compared
- 4) the relationship of diabetic control and erythrocyte filterability was determined

#### 4-2 METHODS

##### 4-2-1 Statistical Analysis

The data from the erythrocyte filtration experiments was analyzed using non-parametric statistical methods because of the distribution of the data. The association

of filterability with various factors was analyzed with Spearman's test of rank correlation, differences between diabetic and normal groups was analyzed with the Mann Whitney U test and the effects of incubation of erythrocytes with insulin on filterability with Wilcoxon's test. Simple linear regression lines were calculated from the data of filtration pressure and the calculated residual count of the suspension.

#### 4-2-2 Erythrocyte Suspension Filtration

Details of the methods are given in chapter 3-3.

### 4-3 RESULTS OF ERYTHROCYTE SUSPENSION FILTRATION IN NORMAL VOLUNTEERS

Fourteen normal non-diabetic volunteer subjects donated blood samples for the erythrocyte suspension studies. The median age was 28.0 years, (range 17 - 62 years). There were 7 males and 7 females. Details of subjects studied and results are given in table 4-1. The median filterability of the dilute erythrocyte suspensions was 0.7 mm Hg., range 0.2 - 2.5 mm Hg. and the median calculated residual leucocytes of the erythrocyte suspensions was  $1.4 \times 10^7/L$ , range  $0.5 - 2.8 \times 10^7/L$ . The results were analyzed for any association between the calculated number of residual leucocytes in the erythrocyte suspension and the filterability of the suspension in mm Hg.. Spearman's test of correlation just failed to reach statistical significance with an Rs of 0.5127,  $p=0.0574$ , (linear regression analysis from the data  $y=0.28 + 0.44x$ ,  $r=0.487$ ,  $n=14$ ,  $p=0.0772$ ).

### 4-4 RESULTS OF ERYTHROCYTE FILTERABILITY IN DIABETIC PATIENTS

Erythrocyte suspension filterability was also examined in 20 insulin requiring diabetic patients of whom 4 were female and 16 male. Seven of the subjects had newly diagnosed diabetes and were examined within a month of diagnosis. The remainder were established diabetics with known disease of more than five years duration. The median age was 42, range 25 - 62 years. Details of patients and results are given in table 4-2. Two patients suffered from background retinopathy, one of whom also had evidence of peripheral neuropathy. One of the newly diagnosed diabetic patients, B.F. had hypertension and moderately impaired renal function secondary to a congenital renal abnormality, and another, L.C., had mild essential hypertension.

Fasting blood glucose levels and  $HbA_{1C}\%$  were estimated on blood sampled at the same time as venesection for the filterability experiments. The median whole blood glucose was 10.1 mmol/l. (range 4.3 - 73 mmol/l) and the median  $HbA_{1C}$  was 11.3% (range 8.7 - 25%). The median filterability pressure was 1.6 mm Hg. (range 0.5 - 9.0 mm Hg). The median calculated residual leucocyte count of the erythrocyte suspension

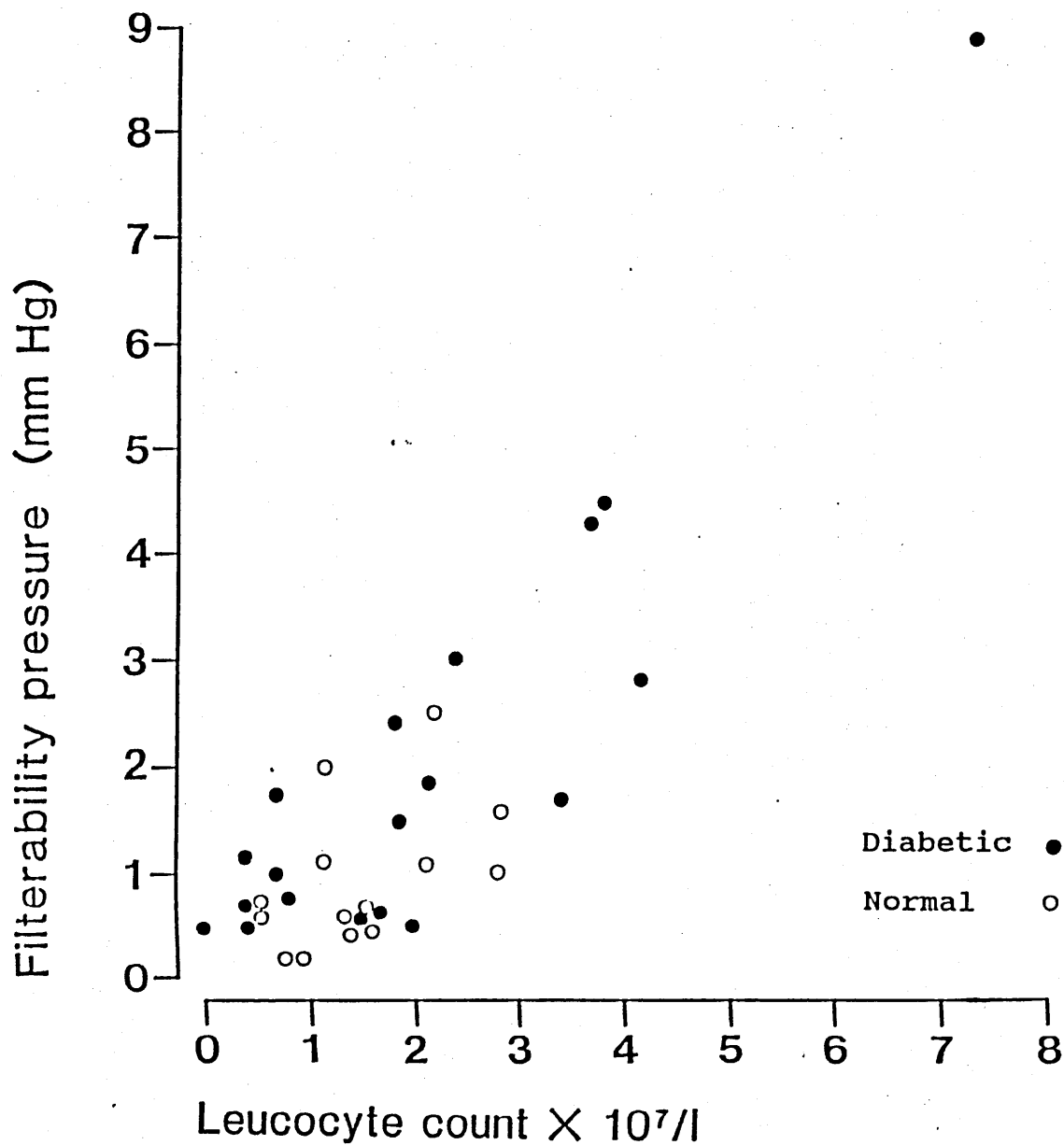
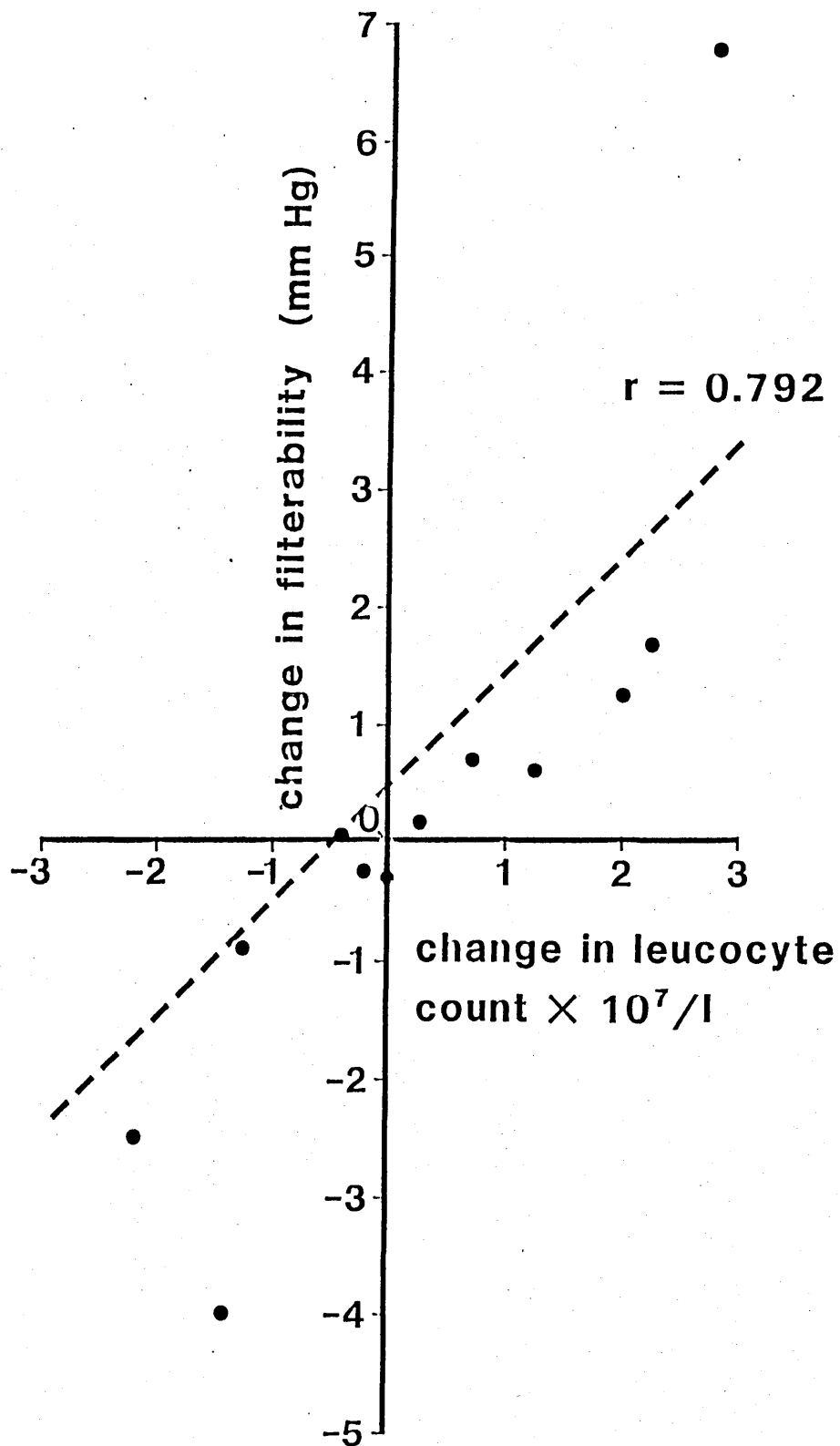


FIGURE 4-1

Graph of the Relationship of Erythrocyte Suspension Filterability and the Calculated Residual Leucocyte Concentration of the Filtered Suspension in Diabetic Patients and in Normal Subjects



**FIGURE 4-2**

The Change in Erythrocyte Suspension Filterability After a Period of Improved Diabetic Control as a Function of The Change in the Calculated Leucocyte Concentration of the Filtered Suspensions



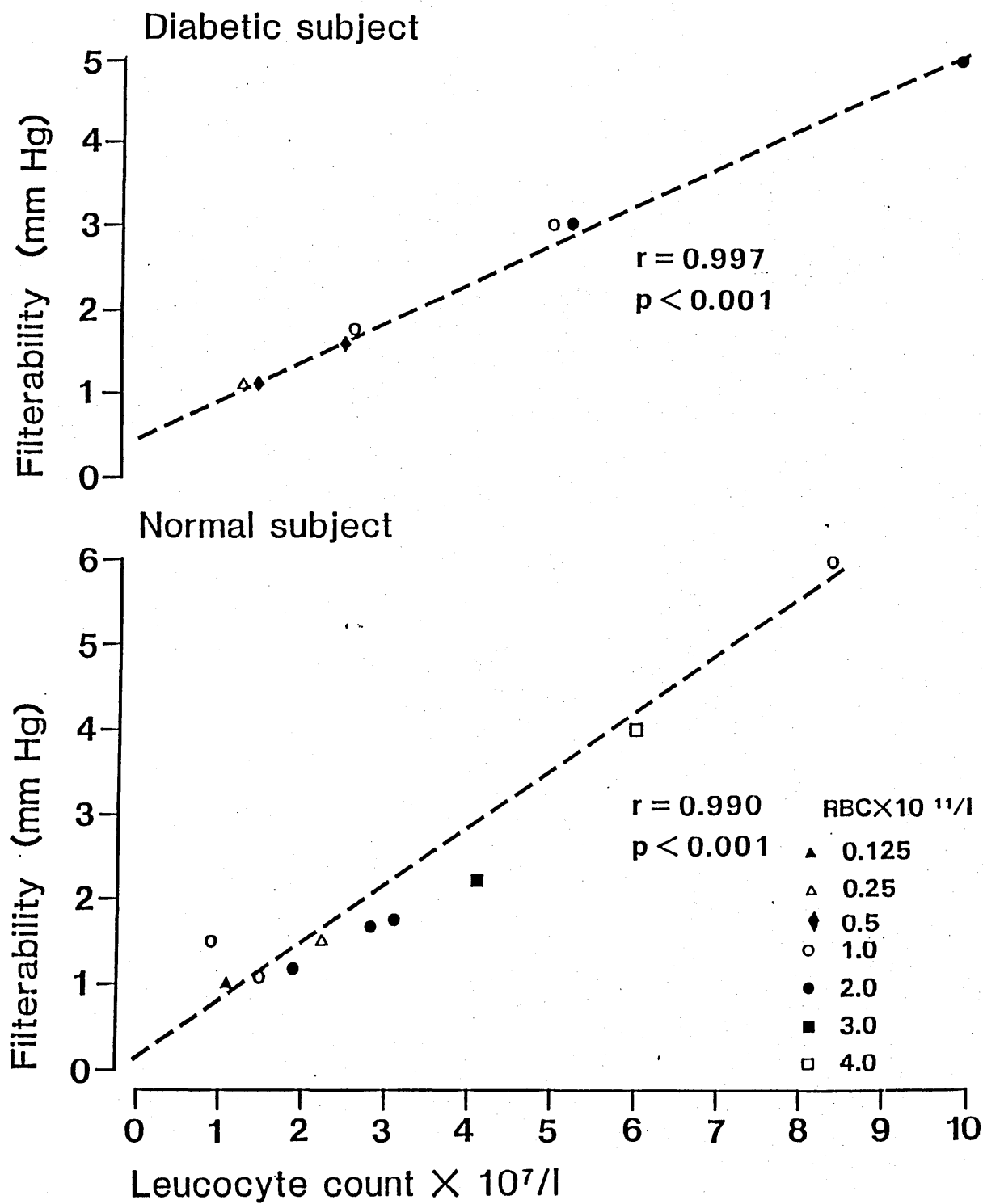
was  $1.7 \times 10^7/\text{L}$  (range 0 -  $7.3 \times 10^7/\text{L}$ ). There was no association between  $\text{HbA}_{1\text{C}}$  levels or whole blood glucose levels and the measured filterability but there was a strong association between suspension filterability and the calculated residual leucocyte count of the suspension, ( $p < 0.0001$ ), see figure 4-1. There was no significant difference between the filterability of normal and diabetic erythrocyte suspensions (see figure 4-1).

#### 4-5 THE EFFECT OF IMPROVED DIABETIC CONTROL ON ERYTHROCYTE SUSPENSION FILTERABILITY

The effect of improved diabetic control was examined in 12 diabetic subjects of whom 6 were newly diagnosed and the rest poorly controlled diabetics who received intensified treatment to improve their blood glucose levels. The time between measurements of filterability varied from 30 - 90 days and in all individuals there was a significant fall in  $\text{HbA}_{1\text{C}}\%$ , median 2.8%, (see table 4-3). There was no significant change in the erythrocyte suspension filterability or any association between the change in  $\text{HbA}_{1\text{C}}\%$  and the change in suspension filterability. Analysis of the relationship between the calculated residual leucocyte count and the suspension filterability, before and after the period of improved diabetic control, showed that the main determinant of filterability was the calculated residual leucocyte count on each occasion. The difference in the filterability between visits one and two correlates positively with the difference of the leucocyte concentration of the suspensions on each occasion (see figure 4-2).

#### 4-6 THE EFFECT OF VARYING THE CONCENTRATION OF LEUCOCYTES AND ERYTHROCYTES ON SUSPENSION FILTERABILITY

To determine the effect of leucocytes on erythrocyte suspension filterability, suspensions of various concentrations of erythrocytes and leucocytes were prepared in the following manner: whole blood samples were centrifuged at 300 G for 30 minutes and the platelet-rich plasma removed. The erythrocytes and buffy layer were resuspended in PBS and then one part of each specimen was pre-filtered over a leucocyte removal filter before washing which increased the removal of leucocytes, whilst the other part was only washed in PBS. The pre-filtered (virtually leucocyte-free) suspensions and the washed cell preparations containing residual leucocytes were made up in different proportions to obtain suspensions of independently varying erythrocyte concentrations,  $1.25 - 40.0 \times 10^{10}/\text{L}$  and leucocyte concentrations,  $1.06 - 10.0 \times 10^7/\text{L}$  (see table 4-4). Normal and diabetic samples were examined. There was no relationship between the erythrocyte concentration of the suspensions and the pressure generated on filtration. However the relationship between the leucocyte concentration and the measured filterability was linear despite an independent variation



**FIGURE 4-3**

The Effects of Varying the Concentration of Leucocytes and Erythrocytes on  
Suspension Filterability  
a) a Diabetic Subject  
b) a Normal Subject

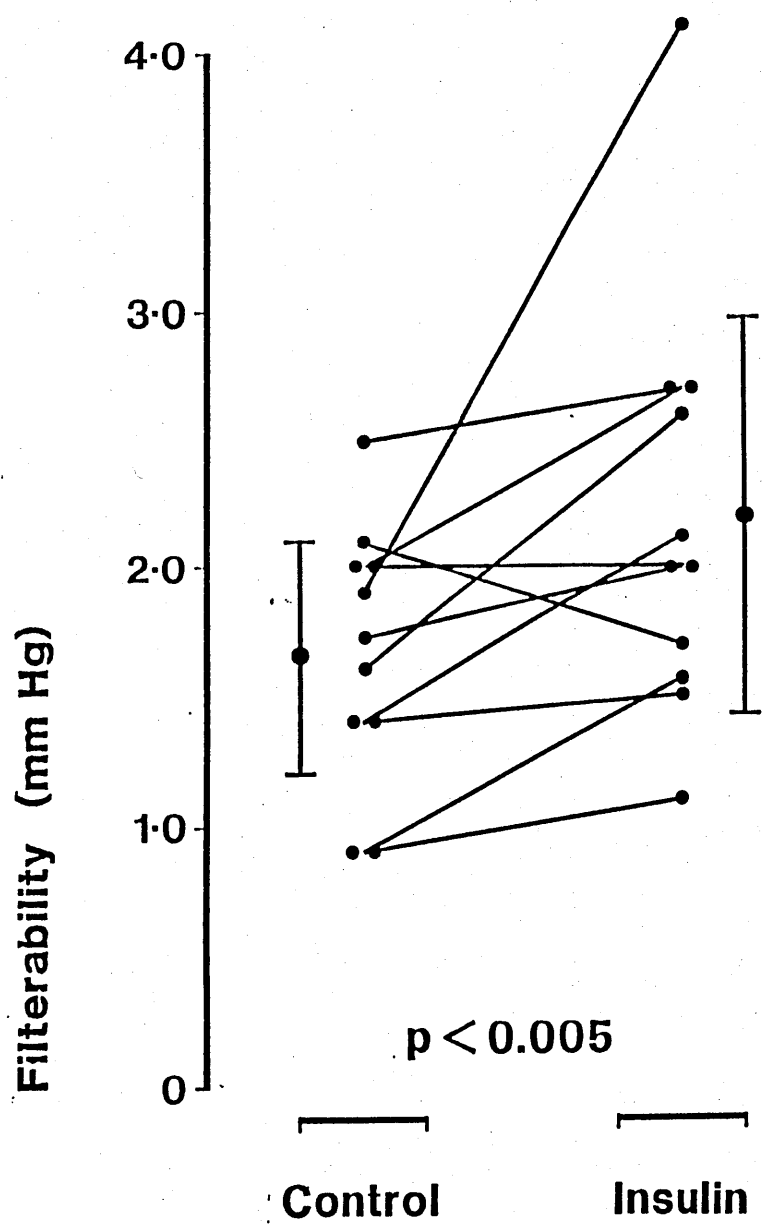


FIGURE 4-4

The Effects of Insulin on Erythrocyte Suspension Filtration

in erythrocyte concentration by a factor of 32 in the normal and a factor of 8 in the diabetic blood samples tested (see figure 4-3).

#### 4-7 THE EFFECT OF INCUBATION WITH INSULIN ON ERYTHROCYTE SUSPENSION FILTERABILITY

The effect of incubation with insulin on erythrocyte filterability was examined in 10 Type II diabetic patients. The buffer used in this set of experiments was Tyrodes with calcium, as calcium may be necessary for insulin to bind to membrane receptors. The erythrocyte suspensions were prepared as described in chapter 3 and after dilution to  $1.0 \times 10^{12}$ /L erythrocytes, the sample was split into two parts, to one of which was added insulin  $10^3$  U/ml and to the other an equivalent volume of buffer. The test and control specimens were drawn up into 20 ml size syringes, capped and placed in an agitating water bath, temperature  $37^\circ\text{C}$ , where they were incubated for one hour. The mean filterability of the control samples was 1.7 mm Hg and that of the insulin treated samples was 2.2mm Hg, an average of 33% less filterability,  $p < 0.0005$ , (see figure 4-4).

#### 4-8 DISCUSSION

##### 4-8-1 The Effects of Leucocytes on the Erythrocyte Filtration System

The main conclusion from this series of cell filtration experiments is that the calculated number of residual leucocytes contaminating the erythrocyte suspension is the major determinant of its filterability. This relationship for the residual suspension leucocytes and filterability was first apparent in the large inter-assay variation of suspension filterability in normal subjects (see Chapter 3-3). These subjects were examined on 3 separate occasions during the course of one week and in each instance the number of contaminating leucocytes in the suspension was different. In repeated examination of erythrocyte suspension filterability in 12 diabetic subjects, (before and after a period of improved diabetic control) in every case the suspension containing the larger number of contaminating leucocytes was less filterable. In diabetic suspension samples there was a linear relationship between filterability and the calculated residual leucocyte concentration, although in normal controls this relationship failed to reach statistical significance. In experiments, where the erythrocyte suspensions were enriched with leucocytes, a linear relationship between the number of residual leucocytes and suspension filterability was found which was independent of the erythrocyte concentration. Whilst, no measurements were made of erythrocytes of known reduced deformability, such as sickle cells (Usami et al, 1975; Chien, 1977), when a suspension of artificially hardened gluteraldehyde treated cells was filtered, the cells were unable to pass through the Nucleopore filter. A rapid rise of filtration pressure occurred. Thus, the erythrocyte filtration test was sensitive to the presence of

hardened erythrocytes, but the contribution of normal and diabetic erythrocytes to measurements of suspension filterability appeared to be small.

The effects of leucocyte interference, even on filtration measurements of dilute erythrocyte suspensions, was suggested by Milligan (1983 [a]), who studied patients with polycythaemia rubra vera and thus high leucocyte counts. The fact that measurements of filterability were made of the rise in pressure occurring during the first 66 seconds of cell filtration suggests that the system was not in equilibrium and that progressive holdup at the filter was occurring. This rate of holdup appears to correspond to the concentration of leucocytes present. Buffer, when filtered, produces a graph of a small initial deflection on starting the system followed by a steady plateau (see Milligan, 1983 [a]). Suspensions with a high residual leucocyte concentration show a similar sharp initial pressure deflection followed by a steady rise of pressure with time, see figure 3-5, which eventually reaches a plateau (not shown in Figure 3-5). It is speculated that this sequence of events might represent a progressive blockage of filter pores by leucocytes. As gradually more filter pores are occupied and fewer are available for entry the rate of rise of pressure decreases. When the pressure over the filter reaches a critical point, it is sufficient to dislodge leucocytes from the filter pores and an equilibrium or plateau of pressure is reached. In the suspensions with low leucocyte concentrations the rate of rise of pressure is slower and the state of equilibrium will take longer to reach and may not be attained. Other workers have postulated that the rate of rise of pressure is a function of the presence of less deformable erythrocytes which also block filter pores (Jones J.G. et al 1984). Stuart et al (1983) have suggested that by using regression line data of the relationship of filterability and the leucocyte concentration, results can be adjusted to a standard leucocyte count, and hence the contribution of erythrocytes to the suspension filterability can be calculated. However, this assumes that leucocyte filterability is constant and not affected by disease states, an assumption which cannot be made due to lack of the appropriate studies. It has also been suggested that below a certain critical level of contamination,  $<0.025 \times 10^9/\text{L}$  (Stuart, 1985) that contamination with leucocytes is unimportant. In this authors experiments, a manipulation of the erythrocyte count by a factor of 32 did not influence the relationship of the rate of rise of the pressure on filtration and the calculated number of residual leucocytes in the suspension. In both normal and diabetic subjects, when virtually all the leucocytes were removed, the pressure generated on filtration suspension was minimal. Normal leucocytes are known to be much less deformable than erythrocytes, (Lichtman, 1973) and in the microcirculation erythrocytes flow in trains held up behind a leucocyte. The in vitro measurements of filterability presented in this thesis appear to be primarily sensitive to the presence of leucocytes, and only when grossly affected by gluteraldehyde do erythrocytes influence readings.

#### 4-8-2 Erythrocyte Deformability in Diabetes Mellitus

Until the early 1980's, it was firmly believed that erythrocyte deformability was reduced in diabetes mellitus, but most of the evidence came from studies of filtration of erythrocytes suspended in plasma, where no particular steps had been taken to avoid the effects of leucocyte contamination or blood proteins (Schmid-Schonbein & Volger, 1976) and from studies of whole blood filtration (Barnes et al, 1977, Juhan et al, 1978). Following presentation of this data at the International Conference of Biorrheology in Baden-Baden in 1983, subsequent publications of studies of erythrocyte suspension filterability have reported that erythrocyte deformability is not reduced in diabetes mellitus, neither on filtration of dilute leucocyte depleted erythrocyte suspensions, (Stuart et al, 1983) nor on examining shear stress induced deformation of erythrocytes (Williamson et al, 1985).

However, it might be argued that the extensive processing of blood samples before filtration tests, which it is now felt essential to carry out, may well have destroyed the least deformable erythrocytes, negating the test. Centrifugation of blood separates the cellular elements of blood by density, with the least dense cells migrating to the upper layer of the centrifugation tube, (Murphy, 1973). Even during careful removal of the buffy layer, small quantities of the least dense cells might be removed. These cells are usually the youngest and have a higher MCV. However young cells are, if anything, more deformable than older cells (Pfafferott et al, 1982; Sutura et al, 1985).

Nevertheless, changes in the average MCV might indicate selective removal of subpopulations of erythrocytes. It is also possible that less deformable erythrocyte are destroyed or retained during passage over leucocyte removal filter. Therefore, evidence of erythrocyte destruction during preparation of erythrocyte suspensions was looked for by comparing the processed erythrocyte MCV with that of whole blood erythrocyte MCV, and by examining the supernatant of erythrocyte suspensions for evidence of haemolysis. In the authors experiments, there was no significant change in the cell size of normal erythrocytes prepared for filtration studies. However, light spectrophotometric measurements of the plasma supernatant of erythrocytes passed over the leucocyte removal filter suggested that some haemolysis had occurred. Thus the processed erythrocytes may not be representative of the original population.

Other recent studies of the use of Leucocyte Removal Filter apparently contradict these findings. The Imugard technique was not found to cause significant retention of subpopulations of glutaraldehyde hardened erythrocytes which were flushed through the Leucocyte Removal Filter (Kenny et al, 1983) nor to affect the density fractionation of cells after passage through a cotton wool filter, (Baar et al, 1986).

#### 4-8-3 New Techniques for Measuring Erythrocyte Deformability

These problems in assessing erythrocyte deformability by dilute erythrocyte suspension filtration techniques have stimulated interest in developing more reliable techniques. The immediate rise in pressure on commencing suspension filtration has been suggested as a more sensitive measure of erythrocyte deformability, (Hanss, 1983). The initial rise in pressure thought to be dependent on several factors including disaggregation of erythrocytes and alignment in the direction of flow and deformation of erythrocytes at the filter pore. The mechanical artifacts of the system used in the studies in this thesis made this initial pressure rise an unreliable parameter. Modifications to the apparatus including addition of a stirring magnet to the suspensions to prevent erythrocyte aggregation and inclusion of a double infusion system of buffer in one line and erythrocyte suspension in the other might have overcome some of these mechanical artifacts by permitting smooth transition from filtration of buffer to filtration of cell suspensions. It should thus be possible to record the rise in pressure due to deformation of cells at the filter but even in this system, it would be necessary to exclude the presence of pore occlusion. In a comparison study of initial flow rate measurements and positive pressure filtration methods for the study of erythrocyte deformability, the final pressure measurements, which this author believes largely represent the presence of occluded filter pores, were found to be more sensitive to the presence of abnormal cells, (Lucas et al, 1983 [b]). This suggests that the initial pressure system may also simply be measuring early pore occlusion. A system which does appear to have overcome the deficiencies of suspension filtration, is the single erythrocyte rigidometer (Kiesewetter et al, 1981) in which a profile of the passage time of erythrocytes is documented. Cells blocking the filter pore can be removed and this system allows a fuller assessment of the whole cell population. Recently, an innovative filtration technique, the St George's Blood Filtrometer as been introduced (Matrai et al, 1984). This filtrometer discriminates between cell transit time and pore occlusion and is reported to distinguish between erythrocyte and leucocyte filtration. Using this new more sensitive technique, Ernst & Matrai (1986) have examined erythrocyte suspension filterability in 23 Type II diabetic patients. They found a small but significant reduction of both erythrocyte and leucocyte filterability in diabetes mellitus.

#### 4-8-4 Conclusions

Given the main conclusions of the leucocyte concentration dependability on filtration pressure, it is not possible to comment on the effects of diabetes mellitus on erythrocyte filterability, other than to note that the leucocyte effect was of similar magnitude in both normal and diabetic subjects, though studies of leucocytes in diabetes mellitus may themselves prove interesting. Experiments of the effect of incubation with insulin on suspension filterability suggested that supra-physiological

concentrations of insulin impaired filterability. It is not possible to say whether this was due to leucocyte or erythrocyte effects. The erythrocyte has no nucleus or protein manufacturing capacity but its membrane has insulin receptors. When insulin binds to a membrane receptor, the insulin-receptor complex is believed to be internalized and in theory changes in the cell membrane may alter membrane fluidity, (Gambhil et al 1978), but the major effects may be on the leucocytes and this requires further investigation.

The evidence, from the studies presented in this thesis, would support the view that there is no gross impairment of erythrocyte deformability in diabetes mellitus. However, the results cast considerable doubt on the sensitivity of positive pressure filtration techniques as a measure of erythrocyte deformability and the normality of erythrocyte deformability in diabetes mellitus requires further confirmation by different methods.



**APPENDIX****TABLES TO CHAPTER 4**

Table 4 -1

## Normal Range of Dilute Erythrocyte Suspension Filterability

Subject	Sex	Age	WCC $10^7/L$	Filterability mmHg
1	F	17	1.6	0.6
2	M	23	0.5	0.6
3	M	28	0.5	0.7
4	F	22	1.4	0.6
5	F	24	1.4	0.4
6	F	62	0.9	0.2
7	F	34	2.8	1.0
8	M	52	1.2	2.0
9	M	23	2.2	1.1
10	M	28	1.6	0.7
11	M	24	2.8	1.6
12	M	31	1.0	0.2
13	F	28	1.2	1.1
14	F	28	2.2	2.5

WCC = The calculated leucocyte concentrations of the suspension  $10^7/L$   
 and Filterability - Rise in pressure on filtering the suspensions at  
 a constant rate of 3.5 mls/minute from 6 - 66 seconds expressed in  
 mm Hg.

Table 4 - 2

Details of the Diabetic Subjects and Erythrocyte  
Suspension Filterability Results expressed in mm Hg.

Subject	Sex	Age	N/E	B.G.	HBA <sub>1</sub> C	WCC	Filterability
NU	F	62	N	5.3	15.1	N.A.	2.0
BE	F	25	N	6.0	11.2	0.44	0.7
CL	F	55	N	13.0	12.5	1.6	0.6
FA	M	44	N	73.0	25.0	0.0	0.5
KI	M	26	N	7.9	9.9	1.7	0.6
LI	M	31	N	8.2	13.1	0.7	1.0
BR	M	44	N	5.7	12.5	1.9	1.5
RC	F	35	E	10.9	10.1	2.2	1.9
RO	M	44	E	8.9	11.6	0.7	1.8
FI	M	40	E	13.1	9.0	3.8	4.4
WH	M	39	E	10.1	11.4	0.4	0.5
MI	M	47	E	13.7	10.7	1.7	2.4
FO	M	55	E	5.2	8.7	0.8	0.8
WA	M	25	E	N.A.	9.4	2.0	0.5
HA	M	62	E	11.4	13.7	0.4	1.2
MD	M	24	E	19.0	12.0	3.4	1.7
WA	M	50	E	14.3	17.3	3.7	4.3
RE	M	35	E	5.5	9.5	2.4	3.0
MA	M	26	E	18.8	10.9	4.2	2.8
SM	M	48	E	10.0	10.0	7.3	9.0

Sex M = male, F = female, N = newly diagnosed, E = established diabetes mellitus, W.C.C. = the calculated leucocyte concentration of the suspension  $10^7/l$  and Filterability = rise in pressure on filtering the suspension at a constant rate of 3.5 mls/minute from 6-66 seconds expressed in mm Hg. N.A. = not available.

Table 4 - 3

The Change in Erythrocyte Filterability between 1) Poor Control of Diabetes Mellitus HbA<sub>1c</sub> >10% and 2) after a Period of Improved Diabetic Control confirmed by a significant fall in HbA<sub>1c</sub>

Subject	Filterability 1-2	WCC 1-2	HbA <sub>1c</sub>
NU	- 0.70	- 0.80	2.3
BE	+ 0.20	+ 0.20	1.9
FA	+ 0.30	0	7.3
KI	- 0.60	- 1.25	1.6
LI	- 6.8	- 2.79	5.2
BR	+ 0.3	+ 1.32	4.3
VH	- 1.7	- 2.26	0.8
HA	- 0.2	- 0.25	3.9
FO	+ 0.4	+ 0.04	1.7
WA	+ 2.15	+ 2.50	5.6
RE	- 2.00	- 1.28	1.5
HE	+ 1.4	- 4.00	3.3

Subject H.E., a newly diagnosed female diabetic patient age 14, is not included in table 4 - 2, as erythrocyte filterability was measured using a different batch number of nucleopore filters. Filterability 1-2 is the filterability of the suspension expressed as mmHg on 1, visit 1 during poor diabetic control, minus the filterability of the suspension (mmHg) on 2, visit 2 after a period of improvement diabetic control. WCC 1-2 is the calculated leucocyte concentration of the suspension  $10^7/L$  on visit 1, visit 1 minus the calculated leucocyte concentration on visit 2, visit 2. HbA<sub>1c</sub> 1-2 is the fall in HbA<sub>1c</sub> % between visits 1 and 2.

Table 4 - 4

Filterability expressed in mm Hg for various Erythrocyte and Leucocyte concentrations  
 Erythrocyte concentration is expressed as R.B.C.  $10^{10}$  /L; Leucocyte concentration as W C C  $10^7$ /L

Subject	Suspension R B C $10^{10}$ /L	Suspension W C C $10^7$ /L	Filterability mm Hg	Filterability (F) as a function of the W C C. of the suspension
a) Male diabetic fasting blood glucose = 21.6 mmol/L	2.5	1.25	1.1	$F = 0.453 + 0.57 \text{ W C C } 10^7/\text{L}$ $p < 0.001$
	5.0	1.3	1.1	
	5.0	2.5	1.6	
	10.0	2.6	1.8	
	10.0	5.0	3.0	
	20.0	5.2	3.0	
	20.0	10.0	5.0	
b) Female normal	1.25	1.06	1.0	$F = 0.066 + 0.682 \text{ W C C } 10^7/\text{L}$ $p < 0.001$
	2.5	2.12	1.5	
	10.0	0.97	1.5	
	10.0	1.5	1.1	
	10.0	8.49	6.0	
	20.0	1.94	1.2	
	20.0	2.8	1.7	
	20.0	3.15	1.8	
	30.0	4.2	2.4	
	40.0	6.0	4.0	

## CHAPTER 5

### WHOLE BLOOD VISCOSITY RESULTS

#### 5-1 INTRODUCTION

The presence of raised whole blood viscosity in association with diabetes mellitus is well documented, but whether the magnitude of these viscosity changes is sufficient to affect blood flow in vivo is unknown. Under abnormal blood flow conditions, where shear rates are low, whole blood viscosity may have an enhanced role in determining blood flow. The increase in whole blood viscosity described in diabetes has been noted to be greatest in vitro when measured at low shear rates (Barnes et al, 1977; McMillan et al, 1980), conditions which might occur in vivo, in the presence of diabetic vascular damage or in the post capillary venules where the first changes of diabetic microangiopathy are found. Under these circumstances, a raised viscosity might serve to increase hypoxia and ischaemia of endothelial cells by causing microvascular sludging, so decreasing local blood flow further and, by increasing intracapillary pressure, thus leading to leakage of serum contents into tissue space. Raised whole blood viscosity has been reported in association with poor diabetic control (Skovborg and Nielsen, 1969; Schmid-Schonbein and Volger, 1976; Paisey et al, 1980) but Isogai et al (1976) have suggested that raised blood viscosity occurs only with diabetic vascular complications. However, the changes of whole blood viscosity described in diabetes are small and although the mean value whole blood viscosity is increased, values are generally not outwith the normal range (Poon et al, 1982) and can be accounted for by a small increase in haematocrit. Whether a raised blood viscosity in diabetes mellitus is an important factor in the development of microvascular damage, or indeed aggravates circulatory insufficiency (see McMillan, 1983) and potentiates vascular damage is still a subject of speculation.

The studies of whole blood viscosity were undertaken to examine the magnitude of blood viscosity changes in diabetes mellitus and to establish whether any significant change could be achieved by therapeutic manipulations, including 1) that of improving diabetic blood glucose control and 2) the influence of treatment with biosynthetic human insulin. At the outset of these studies, little was known of the therapeutic gains to be obtained from treatment with biosynthetic human insulin but it was hoped that this hormone would prove less immunogenic than animal insulins and possible effects on plasma proteins might influence whole blood viscosity in diabetes mellitus. Lastly, it was hoped to establish if whole blood viscosity was related to peripheral blood flow in vivo in diabetic patients.

## 5-2 AIMS OF THE STUDIES

The aims of the studies of whole blood viscosity in diabetes mellitus were as follows:

- a) to establish a normal range of whole blood viscosity at low shear rates  $2.62 \text{ secs}^{-1}$  and  $0.130 \text{ secs}^{-1}$
- b) to measure whole blood viscosity in diabetic patients and to determine the relationship with blood haematocrit and fibrinogen levels
- c) to determine the effects of improving diabetic control on whole blood viscosity
- d) to establish whether treatment with biosynthetic human insulin had any effect on whole blood viscosity in diabetic patients
- e) to establish whether there is any relationship between whole blood viscosity and peripheral blood flow in the lower limb in diabetes mellitus.

## 5-3 DETAILS OF THE NORMAL CONTROL AND THE DIABETIC SUBJECTS STUDIED

### 5-3-1 Normal Control Subjects

Twenty normal control subjects, 7 females and 13 males, age range 24 - 65 years, were studied, all of whom were healthy and gave no history of previous illness. Details of this group are given in Table 5-1.

### 5-3-2 Diabetic Patients

Twenty-one diabetic subjects, consisting of the 15 established diabetic patients who took part in the double-blind study of treatment with BHI and PPI (details of whom are supplied in Table 6-3) and 6 Type II patients with evidence of microangiopathy (details of whom are given in Table 5-2) were studied. There were 14 males, 7 females and the median age was 52, range 27 - 68 years. Results of whole blood viscosity are given in table 5-3. The characteristics of the normal control subjects and diabetic patients are summarized in Table 5-4.

## 5-4 METHODS AND STATISTICAL ANALYSIS

### 5-4-1 Methods

Details of the Whole Blood Viscosity method are given in Chapter 3-2. The normal control subjects attended the Department of Medicine to donate post prandial blood samples for the viscosity measurements, because this proved logistically easier. The

diabetic patients were all studied fasting before any morning medication at entry to the study and after a 3 month period of attempted improved diabetic control achieved through intensive advice and manipulation of their usual treatment, see chapter 2-3 for details of management. Samples for haematocrit, fibrinogen whole blood glucose and HbA<sub>1c</sub> levels were taken at the time of sampling for viscosity measurements. The 15 insulin requiring diabetics who participated in the double-blind study of treatment with purified porcine and biosynthetic human insulins were studied after 3 months treatment with each of the trial insulins and were managed as described in chapter 2-4. These patients were thus examined 4 times 1) at entry to the study, 2), after a run-in period of optimized diabetic control and 3) and 4) after treatment with BHI and PPI respectively. Unfortunately, because of equipment failure, data for the effects of improved control during intensified normal treatment are only available on 12 patients (3 Type II and 9 Type I) and of treatment with BHI and PPI in 14 patients. The relationship of peripheral blood flow and whole blood viscosity was examined in 13 normal subjects and in all 21 diabetic patients. Full details of the measurements of foot flow are given in chapter 3-5. Blood for the viscosity measurements was collected one hour before the blood flow measurements and viscosity was measured within 3 hours.

#### 5-4-2 Statistical Analysis

The whole blood viscosity results were analyzed using non- parametric statistical methods because of the distribution of the data. In the discussion section, the results are also expressed as mean values, to allow comparison with other published data. The diabetic subjects were compared with normals using the Mann Whitney U test and the relationships between whole blood viscosity with diabetic control and plasma fibrinogen were examined using Spearman's test of rank correlation. Linear correlation analysis was used to examine the relationship of the logarithm of viscosity with haematocrit. The effects of improved diabetic control on whole blood viscosity were examined using the Wilcoxon rank signed test. In order to eliminate the influence of any sequential trend throughout the double-blind crossover study of BHI and PPI, the change in whole blood viscosity which occurred on transfer to each insulin (compared with the immediately preceding measurement of blood viscosity) was analyzed using Wilcoxon's rank signed test.

### 5-5 RESULTS

#### 5-5-1 Comparison of Normal Volunteers and Diabetic Subjects

Results of the whole blood viscosity measurements and sample haematocrit and fibrinogen levels on individual normal subjects and diabetic patients are shown in Tables 5-1 and 5-3 respectively and summarized in Table 5-4. In both the controls



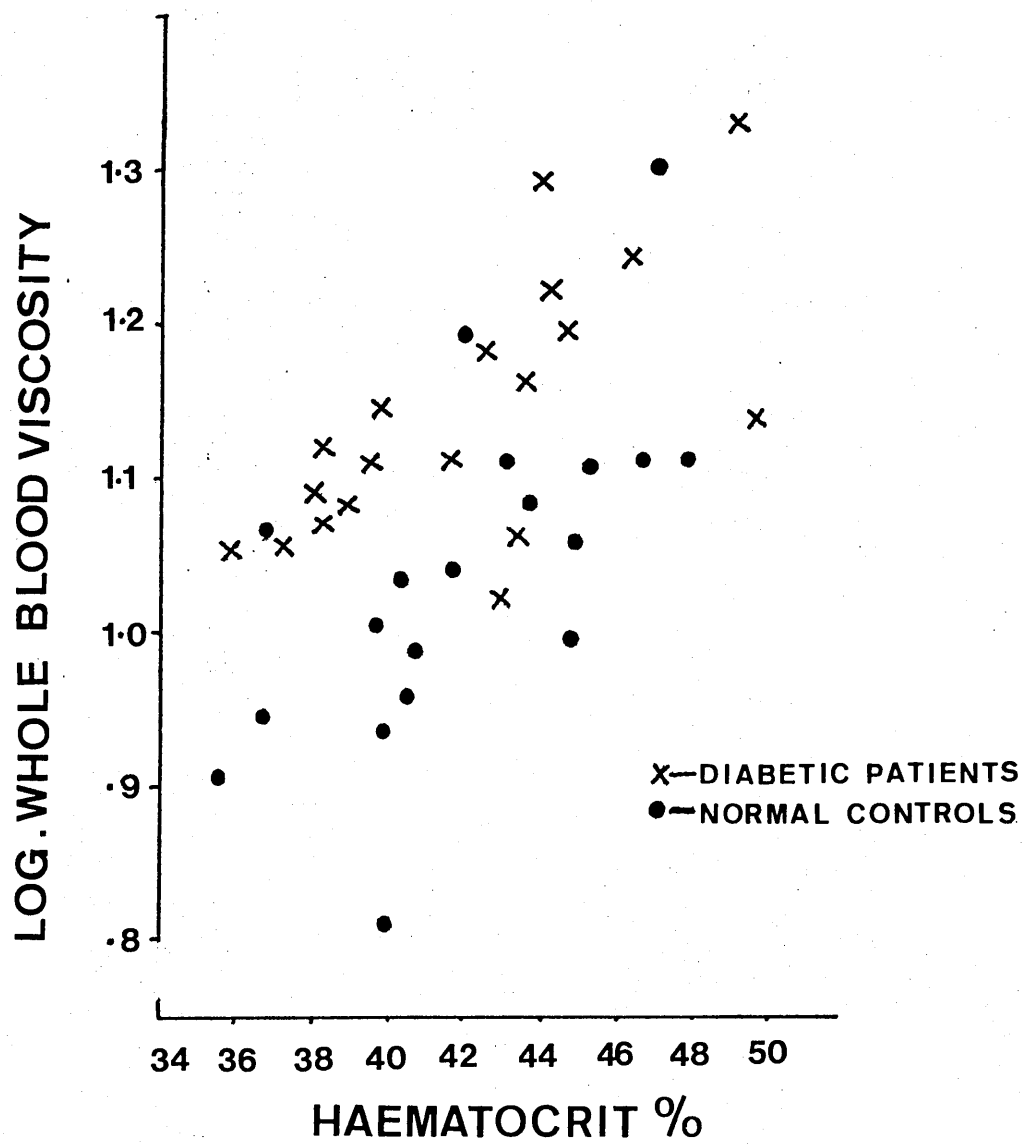


FIGURE 5-1

Graph of the Relationship of Whole Blood Viscosity and Haematocrit

and the diabetic patients haematocrit was the most important predictor of whole blood viscosity. There was a linear relationship of the logarithm of whole blood viscosity measured at a shear rate of  $2.62 \text{ secs}^{-1}$  and haematocrit, ( $p < 0.01$  in each case, see Figure 5-1). At the lower shear rate  $0.130 \text{ secs}^{-1}$ , this relationship was weaker in the control group ( $p = 0.0183$ ) and did not reach significance for the diabetic patients. For any given haematocrit, whole blood viscosity measured at shear rate,  $2.62 \text{ secs}^{-1}$  was significantly higher in the diabetic patients, median value 13.3 cp versus control group, median value 11.2 cp ( $p = 0.0029$ ). Fibrinogen was shown to be higher in the diabetic patients, median 2.24gm/l, than in the control subjects, median 1.85 gm/l ( $p=0.05$ ) but fibrinogen levels bore no relationship to whole blood viscosity measurements.

#### 5-5-2 The Effect of a Period of Improved Diabetic Control on Whole Blood Viscosity

A period of 12 weeks of improvement of diabetic control, confirmed by a small but significant fall in  $\text{HbA}_{1\text{C}}$  % from 10.6% to 9.5%, was studied in 12 patients. This did not result in any significant change in whole blood viscosity or changes in fibrinogen levels or haematocrit, (see Table 5-5).

#### 5-5-3 The Results of the Double-Blind Crossover Study of Treatment with Biosynthetic Human Insulin and Purified Porcine Insulin

Whole blood viscosity at shear rate  $2.62 \text{ secs}^{-1}$  was essentially unchanged on treatment with BHI, median change BHI - 0.68 cp compared with a median change on treatment with PPI of - 0.90 cp. There was no significant differences of change in whole blood viscosity measured at  $0.130 \text{ secs}^{-1}$ , median change BHI +3.54 cp, median change PPI -0.31 cp.  $\text{HbA}_{1\text{C}}$ , haematocrit and fibrinogen levels were not significantly different on treatment with BHI compared with treatment with PPI, (see Table 5-6 ).

#### 5-5-4 Relationship of Whole Blood Viscosity to Peripheral Blood Flow:

The whole blood viscosity measurements of 13 normal volunteers and 21 diabetic subjects were examined statistically to see if there was any relationship of blood viscosity measurements to peripheral blood flow in the lower limb as measured by venous occlusion plethysmography. Spearman's test of correlation was performed on viscosity measurements and resting foot flow, time to peak flow ,peak post stimulus (calculated as the measured peak post stimulus flow - the basal resting flow) and the half life of reactive hyperaemia values (see Table 5-7). In the normal control subjects there was a <sup>Negative</sup> correlation between resting foot flow and whole blood viscosity measured at shear rate  $0.130 \text{ secs}^{-1}$ , ( $p=0.0108$ ) but this association

just failed to reach statistical significance at shear rate  $2.62 \text{ secs}^{-1}$ , ( $p=0.0709$ ). In the diabetic patients there was no association with resting foot flow and whole blood viscosity measured at either shear rate. A statistical relationship between the duration of reactive hyperaemia and whole blood viscosity at shear rate  $0.130 \text{ secs}^{-1}$  just failed to reach significance in both the normal ( $p = 0.0568$ ) and diabetic subjects ( $p= 0.0736$ ). There was no association of time to peak flow values and the peak post stimulus flow values with whole blood viscosity. Blood flow results are given fully in chapter 7

## 5-6 DISCUSSION

These studies have shown, as is well recognized (Begg and Hearn, 1966), that the logarithm of measurements of whole blood viscosity is proportional to haematocrit. However, at comparable haematocrit, whole blood viscosity is higher in diabetic patients, as previously described (Skovborg et al, 1966; Hoare et al, 1976; Barnes et al, 1977; Lowe et al, 1980; Poon et al, 1982). Shortterm improvement of diabetic control, as can be achieved by close outpatient supervision had no discernible effects on whole blood viscosity measurements. Neither did treatment with BHI or PPI have any effect on whole blood viscosity. There is an apparent relationship of resting foot flow and whole blood viscosity in normal subjects, but not in diabetic patients.

### 5-6-1 The Physiological Significance of Alterations of Whole Blood Viscosity

When the complex interaction of factors affecting blood flow in the circulation is considered, it is difficult to extrapolate the in vivo dynamic effects of alterations in whole blood viscosity as measured in vitro. In vitro measurements of whole blood viscosity are determined by plasma factors, the number and properties of erythrocytes and other cellular elements, and the temperature of the sample at the time of analysis, (as reviewed by Stuart and Kenny 1980). Plasma viscosity is primarily a function of the plasma protein concentration. Protein molecules behave as rigid particles in solution and their effective volume does not change with increasing shear - thus plasma is a Newtonian solution. Among plasma proteins, fibrinogen, which is a molecule with a large axial asymmetry is a major component in determining viscosity (Usami, 1982). Another major contribution of the plasma proteins to whole blood viscosity is by their interaction with the erythrocytes. Large macromolecular proteins, which are absorbed onto erythrocyte surfaces, can form bridges between the cells leading to the formation of erythrocyte aggregates (Chien and Jan 1973). Fibrinogen is particularly effective in producing erythrocyte aggregation as its large absorbed surface increases the surface absorptive force and the longer inter-cellular distance decrease the electrical repulsive forces

between cells. Cell aggregation is also dependent on the properties of the erythrocyte and is counteracted by mechanical shearing. Erythrocyte aggregation only appears to be important at low flow rates. At low shear rates the rouleaux behave like rigid rods but at higher shear rates, they bend and break up leading to a progressive fall in blood viscosity. Cell aggregation is also limited by vessel size. In vessels wider than erythrocytes, a three dimensional network of aggregated erythrocytes may form leading to an increase in the effective cell volume. In small vessels, only slightly larger than the erythrocyte, rouleaux formation is limited in a linear fashion, aligned with flow, thus decreasing the effective cell volume and viscosity (as reviewed by Usami, 1982).

In vitro, erythrocytes are numerically the most important of the cellular elements determining blood viscosity and blood viscosity, when transformed logarithmically, is linearly related to the blood haematocrit (Begg and Hearn 1966). In vivo, blood haematocrit is a less important predictor of viscosity in the microcirculation, where vessel size plays an important role in determining viscosity. In small vessels, of less than 300  $\mu\text{m}$ , the haematocrit is only 15-20%, and the apparent viscosity is lower than in large vessels where the haematocrit is 45%. This well known phenomenon, described by Fahreus and Lindquist in 1931, is the result of erythrocytes flowing preferentially in the centre of the tube where they can travel faster within the layer of plasma lining the vessel wall. In vessels of less than 29  $\mu\text{m}$  the haematocrit is further decreased as a result of relatively fewer erythrocytes being deformed and entering these smaller capillaries. In vessels which are less than the diameter of the erythrocyte, considerable cell deformation is required before the cells can pass through the vessel. Under normal flow conditions in these small capillaries, high velocity results in high shear stress at the cell surface causing the necessary erythrocyte deformation but the less deformable leucocytes appear to play an important regulatory role (as reviewed by Schmid-Schonbein 1981). Erythrocytes are held up behind the leucocyte, with a red cell depleted region forming downstream of the leucocyte. On entering a wider vessel, the erythrocytes push past the leucocyte pushing it against the endothelium and it is prevented from re-entering the central stream by the continuing stream of erythrocytes. Nucleated leucocytes are much less deformable than erythrocytes and when their number is pathologically raised, as in leukaemia, hyperviscosity syndrome can result (Lichtman, 1973)

In normal flow conditions shear rates are lowest in the post capillary venules and small veins but viscosity may not be much higher than in the post capillary segment as shear rates are still high. In low flow states, relative blood viscosity is increased because of erythrocyte aggregation and lack of shear induced erythrocyte deformation. This results in a much higher viscosity in the post capillary venule

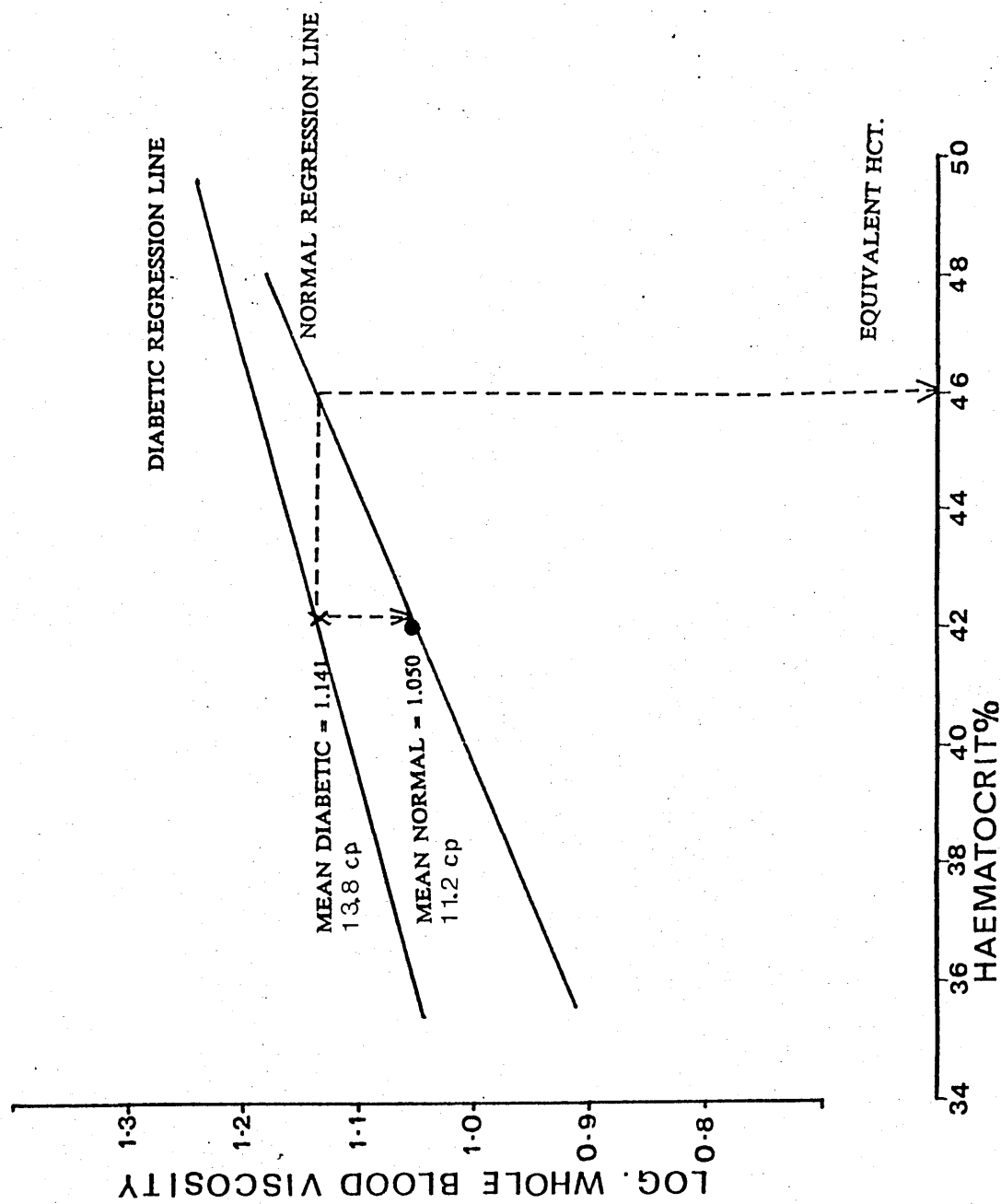


FIGURE 5-2

Regression Lines of Whole Blood Viscosity and Haematocrit

and increased capillary pressure. However in low flow states leucocytes also adhere to the endothelium and it is this factor which is thought to result in the massive elevation in intravascular resistance seen with low flow. This increase in resistance is accentuated if haematocrit is also increased. Counteracting the effects of high haematocrit is the red cell screening effect whereby at low shear rates fewer erythrocytes are sufficiently deformed to enter the smaller branch vessels and hence the relative viscosity is lowered (as reviewed by Usami 1982).

#### 5-6-2 Whole Blood Viscosity and Diabetes Mellitus

Alterations of many of the determinants of whole blood viscosity have been reported in diabetes mellitus. Erythrocyte aggregation and fibrinogen levels are reported as being increased, erythrocyte deformability as decreased and these changes may account for the more marked increase of whole blood viscosity noted in vitro at low shear measurements in diabetes mellitus (Barnes et al, 1977; MacMillan et al, 1980). In the studies presented in this thesis, measurements of whole blood viscosity were made at exceptionally low shear rates, such as might occur in hypoperfused diseased vessels. Whole blood viscosity was increased in the diabetic patients. The uncorrected (for haematocrit) median value was 17.8% higher, when measured at a shear rate of  $2.62 \text{ secs}^{-1}$  than that of normal control subjects. To compare this data with other published studies, mean values were calculated and regression lines of the relationship of whole blood haematocrit and the logarithm of whole blood viscosity was plotted for both diabetic and normal subjects (Figure 5-2). By extrapolation from Figure 5-2, it can be seen that the mean whole blood viscosity of the diabetic patients was approximately 23.0% higher than would have been anticipated for normal subjects with similar mean haematocrits. These results are comparable to those of Lowe et al (1980) whose data give an 11.2 % increase in corrected whole blood viscosity at low shear in 14 diabetic patients without retinopathy and a 16.6% increase in 14 diabetic patients with retinopathy.

By projection from the linear regression lines in figure 5-2, it can be seen that the difference in mean whole blood viscosity between normal and diabetic subjects could be accounted for by a small overall increase in haematocrit of the order of 4%. Similar findings are those of Poon et al, (1982), who reported a 12.8% increase in mean low shear whole blood viscosity in diabetes mellitus but suggested that these changes could be produced by only a 1.7% increase in packed cell volume. It seems unlikely that changes, equivalent to a normal variation in haematocrit, will have any significant effects on blood flow in the microcirculation.

Measurements of whole blood viscosity at the lower shear rate of  $0.130 \text{ secs}^{-1}$  were much more widely distributed and the relationship of viscosity with blood haematocrit was much weaker. Whilst this may reflect on different factors influencing viscosity at this very low shear rate, it seems more likely that mechanical problems simply introduced a greater degree of error. The CV for viscosity measurements at  $0.130 \text{ secs}^{-1}$  ranged from 4.7 - 13.7% compared with a CV of 0.96% for viscosity measurements at shear rate  $2.62 \text{ secs}^{-1}$ .

The failure to demonstrate a consistent improvement of whole blood viscosity with improvement of diabetic control may well reflect on the small overall improvement of diabetic control achieved and the small number of patients studied. The main determinants of viscosity, the haematocrit and the plasma fibrinogen did not change during the period of improved control nor during treatment with BHI. Treatment with BHI had no significant effects on whole blood viscosity. These results are in agreement with Leiper et al, (1984) who also found no difference in whole blood viscosity on treatment with BHI.

The relationship of whole blood viscosity with blood flow was examined in the foot where a large component of distension on venous occlusion plethysmography is due to skin blood flow as opposed to the calf, where measurements are predominantly of muscle flow. There was a statistically significant relationship of higher rates of resting foot flow and lower blood viscosity in normal subjects. However significant associations of blood flow and viscosity were only noted at the less accurately recorded lower shear rate of  $0.130 \text{ secs}^{-1}$  measurement (see section 3-2-3) and the total numbers examined were small. There was no relationship of resting flow rates and blood viscosity in diabetic subjects. In both normal and diabetic subjects there was a trend, which just failed to reach statistical significance, of longer half life of reactive hyperaemia following arterial occlusion and higher whole blood viscosity. Other measures of reactive hyperaemia, such as the time taken to attain peak flow values and the peak post stimulus flow value, did not show any relationship with blood viscosity. Prolongation of reactive hyperaemia following ischaemic stress is a feature of vascular insufficiency; it takes longer to recover from ischaemia when the vascular supply is impaired. However the capacity of the vasculature to vasodilate and the presence of large vessel disease are other important determinants of blood flow and reactive hyperaemia. The associations of blood flow and whole blood viscosity described here, although interesting, require further confirmation before suggesting that minor variations in whole blood viscosity such as occur in diabetes, might significantly influence blood flow in vivo. (Blood flow results are described in detail in Chapter 7)

### 5-6-3 Conclusions

In these studies a small increase in whole blood viscosity in diabetes mellitus, equivalent to a 4.0% increase in haematocrit has been noted but no relationship between foot blood flow and this mild hyperviscosity has been demonstrated. No special effects of treatment with BHI on whole blood viscosity were noted. Future studies of whole blood viscosity in diabetes mellitus might be best advised to address the problem of determining the physiological significance of whole blood viscosity changes in diabetes mellitus.



**APPENDIX****TABLES TO CHAPTER 5**

TABLE 5 - 1

## DETAILS OF NORMAL CONTROL SUBJECTS PARTICIPATING IN WHOLE BLOOD VISCOSITY STUDIES

		RB	DP	PHr	JP	PW	JB	CW	PCo	PL	MLe	RD	JL	RH	LS	DR	GL	SJ	ML	RC	MT
Sex	M/F	M	N	M	M	M	M	M	M	M	M	M	M	M	F	F	F	F	F	F	F
Age	Years	24	28	30	31	32	32	43	47	48	48	50	65	62	29	29	44	48	48	49	58
Weight Index	%	22	23.0	19.2	21.7	18.3	22.0	20.7	24.7	24.9	20.0	24.2	24.0	28.0	26.0	19.5	19.8	23.1	23.6	21.7	26.5
Blood Pressure	mmHg	120/70	125/80	100/60	110/70	120/70	120/80	105/72	130/90	110/60	120/90	120/80	160/100	140/98	120/80	100/60	120/80	120/70	130/65	130/80	150/100
Haematocrit	%	45.0	40.8	40.7	44.8	43.9	43.4	40.4	45.3	40.0	36.2	46.8	48.0	47.3	42.2	36.7	39.9	35.6	NA	41.9	39.7
Fibrinogen	gms/L	1.64	1.55	NA	1.45	NA	NA	1.51	1.87	1.42	1.99	1.93	NA	2.48	NA	NA	1.28	2.26	1.99	1.93	2.5
Viscosity	cp																				
(a) Shear rate	0.130 sec <sup>-1</sup>	43.3	NA	48.8	43.9	59.8	72.0	36.0	57.3	40.2	53.5	62.8	58.4	73.2	63.4	54.9	26.2	41.2	47.9	54.7	55.2
(b) Shear rate	2.62 sec <sup>-1</sup>	11.4	9.6	11.0	9.9	12.2	15.5	9.1	14.3	8.6	11.5	13.6	13.6	20.1	15.6	8.9	6.6	8.0	9.9	11.1	11.4
Logarithm of viscosity measurement	0.130 sec <sup>-1</sup>	1.636	NA	1.688	1.625	1.777	1.857	1.556	1.758	1.604	1.728	1.798	1.766	1.864	1.802	1.740	1.418	1.615	1.680	1.738	1.742
Logarithm of viscosity measurement	2.62 sec <sup>-1</sup>	1.057	0.982	1.041	0.997	1.086	1.194	0.959	1.115	0.937	1.060	1.133	1.133	1.302	1.194	0.949	0.819	0.903	0.996	1.045	1.057

TABLE 5 - 2

DETAILS OF ADDITIONAL DIABETIC PATIENTS PARTICIPATING IN WHOLE BLOOD VISCOSITY STUDIES

	JQ	GM	JS	MD	MW	FW
Sex	M	M	M	F	F	F
Age	59	64	68	45	53	62
Weight Index	22.5	24.2	29.7	24.3	34.8	23.7
Blood Pressure	140/80	200/100	160/90	140/80	190/125	120/70
Duration of Diabetes	2	19	3	8	3	17
Retinopathy	Proliferative	Background	Background	Background	Background	Proliferative
Other diabetic complications						
Treatment for diabetes mellitus	Glibenclamide	Chlorpropamide	Glibenclamide	Glibenclamide	Metformin	Metformin
Other medical conditions	None	Hypertension	None	Menopausal symptoms	Mild hypercalcaemia (primary hyperparathyroidism) Depression	
Other medication	None	Methyldopa amiloride hydrochlorothiazide	None	Oestrogen/ valerate	Amitryptilline	None
Cigarette consumption	0	0	0	0	0	0

TABLE 5 - 3  
WHOLE BLOOD VISCOSITY RESULTS OF DIABETIC PATIENTS

	CS	GT	RK	CB	PF	DC	AE	CH	RC	HW	PP	WM	WH	MT	DD	JQ	CN	JS	YD	Whe	FV
Hemoglobin A <sub>1</sub> C	8.6	10.8	10.4	7.8	9.7	12.6	10.6	10.4	11.8	13.8	13.5	8.5	11.6	12.2	8.7	7.5	8.4	7.5	8.6	6.5	11.6
Hematocrit	39.4	44.7	39.5	48.9	44.0	38.0	38.9	43.6	43.6	46.2	38.2	38.2	41.7	39.7	42.7	42.9	49.7	48.3	37.4	44.2	35.4
Fibrinogen gms/L	2.81	2.38	3.45	2.26	2.36	3.39	2.27	1.75	1.18	2.32	2.55	1.59	2.40	2.74	1.44	2.20	2.63	2.95	2.73	2.54	NA
Viscosity centipoise																					
1) Shear rate 0.130 sec <sup>-1</sup>	42.6	59.3	63.9	76.6	69.0	56.7	54.8	54.0	45.4	78.3	49.0	65.3	74.4	49.4	70.6	NA	48.8	40.6	42.7	71.5	NA
2) Shear rate 2.62 sec <sup>-1</sup>	14.1	15.6	13.2	21.4	19.8	12.3	11.9	14.5	11.6	17.6	13.3	12.0	13.1	12.4	15.2	10.5	13.7	NA	11.4	16.8	11.4
1) Logarithm of viscosity (0.130)	1.630	1.773	1.806	1.864	1.839	1.754	1.739	1.732	1.657	1.894	1.714	1.815	1.872	1.644	1.849	NA	1.688	1.609	1.630	1.854	NA
2) Logarithm of viscosity (2.62)	1.149	1.193	1.121	1.330	1.297	1.090	1.076	1.161	1.064	1.245	1.124	1.079	1.117	1.093	1.182	1.021	1.140	NA	1.058	1.225	1.057

TABLE 5 - 4

## WHOLE BLOOD VISCOSITY RESULTS (MEDIAN AND RANGES)

Subjects	No.	M	F	Age years	Weight Index %	Fibrinogen gm/L.	Haematocrit %	Viscosity (cp) 0.130 secs - 1 2.62 secs - 1
Diabetic Patients	21	14	7	52.0 (27.0-68.0)	24.2 (19.5-34.8)	2.24 (1.46-3.68)	42.7 (35.4-49.7)	56.7 (40.6-78.3) 13.3 (10.5-21.4)
Normals	20	13	7	45.5 (24.0-65.0)	22.5 (18.3-28.0)	1.85 (1.28-2.50)	41.9 (35.6-48.0)	54.7 (26.2-73.2) 11.2 (6.6-20.1)

TABLE 5 - 5

RESULTS OF A PERIOD OF IMPROVED BLOOD GLUCOSE  
CONTROL ON WHOLE BLOOD VISCOSITY (Median and Range)

Median (range)	Before	After
Haemoglobin A <sub>1</sub> C %	10.6 (8.4 - 13.8)	9.5 (7.8 - 13.4)
Haematocrit %	43.6 (37.2 - 49.7)	42.9 (37.4 - 49.8)
Fibrinogen gm/l	2.34 (1.44 - 3.39)	2.25 (1.46 - 3.68)
Viscosity (0.130 secs <sup>-1</sup> ) cp	59.4 (48.8 - 78.3)	62.6 (42.7 - 80.5)
Viscosity (2.62 secs <sup>-1</sup> ) cp	13.9 (10.3 - 21.4)	14.1 (9.7 - 21.9)

TABLE 5 - 6

THE RESULTS OF TREATMENT WITH BH1 AND PP1 ON WHOLE BLOOD  
 VISCOSITY (i) MEDIAN AND RANGE (ii)  $\Delta$  MEDIAN AND RANGE OF  
 OF THE CHANGE OCCURRING ON TRANSFER TO EACH TREATMENT

Median (Range)	BH1	PP1
Haemoglobin A <sub>1</sub> C %	10.4 (8.1 - 11.9)	10.1 (7.0 - 11.7)
Haematocrit %	41.1 (38.6 - 49.6)	42.4 (26.7 - 49.8)
Fibrinogen gm/1	2.01 (1.54 - 3.49)	2.07 (1.17 - 3.66)
Viscosity (0.13 secs <sup>-1</sup> ) cp	57.0 (43.9 - 101.3)	59.7 (28.1 - 92.1)
Viscosity (2.62 secs <sup>-1</sup> ) cp	13.3 (9.5 - 18.1)	12.3 (9.9 - 19.8)

Median (Range)	$\Delta$ BHI	$\Delta$ PPI
$\Delta$ Haemoglobin A <sub>1</sub> C %	+ 0.6 (-2.4 - +3.9)	- 0.1 (-1.4 - +0.8)
$\Delta$ Haematocrit %	- 0.9 (-4.6 - +2.1)	+ 0.3 (-12.4 - +1.4)
$\Delta$ Fibrinogen gm/1	+ 0.02 (-1.2 - +0.59)	- 0.03 (-1.10 - +1.36)
$\Delta$ Viscosity (0.13 secs <sup>-1</sup> ) cp	+ 3.54 (-23.79 - +41.27)	- 0.31 (-29.28 - +27.45)
$\Delta$ Viscosity (2.62 secs <sup>-1</sup> ) cp	+ 0.68 (-3.99 - +6.85)	- 0.90 (-9.4 - +1.73)

TABLE 5 - 7

SPEARMAN'S TEST OF RANK CORRELATION OF WHOLE BLOOD VISCOSITY  
AND PERIPHERAL BLOOD FLOW (p values)

	Whole Blood Viscosity Shear Rate 0.130 secs <sup>-1</sup>	
	Normal	Diabetic
RFF	0.0108	0.7651
TPF	0.9000	0.4026
PPSF	0.3084	0.8875
T50	0.0568	0.0736

	Whole Blood Viscosity Shear Rate 2.62 secs <sup>-1</sup>	
	Normal	Diabetic
RFF	0.0709	0.7492
TPF	0.4233	0.9911
PPSF	0.4810	0.9844
T50	0.1482	0.2340



## CHAPTER 6

### HAEMOSTATIC FUNCTION RESULTS

#### 6-1 INTRODUCTION

Abnormalities of the haemostatic system in diabetes mellitus have been postulated as leading to a hypercoagulable state which, in turn, has been implicated as a causative factor in the development of diabetic vascular complications. But it has not yet been established whether the abnormalities of haemostatic factor levels previously described are a cause, a consequence of, or are irrelevant to the development of diabetic vascular damage. Whilst there is general agreement that activities of the factor VIII complex and fibrinogen levels are increased in diabetes mellitus, there is at present confusion as to the influence of metabolic control, treatment and vascular disease on these haemostatic variables. Furthermore, studies of the fibrinolytic system have provided apparently inconsistent results of normal, raised and lowered activity in diabetes mellitus. Whilst treatment with insulin has been reported to affect the fibrinolytic system and with rebound shortening of euglobulin clot lysis times (Fearnley et al, 1959), it is not known if these effects are directly attributable to insulin or mediated indirectly through its metabolic effects.

#### 6-2 AIMS OF THE STUDIES

The studies of haemostatic function were aimed to establish:

- i) normal ranges for the haemostatic factors under study and the relationship of factors with age, obesity and blood pressure
- ii) the effect of the presence of diabetes mellitus on haemostatic function and the relationship with age, obesity and blood pressure compared with that in normal healthy volunteers
- iii) the effect of the improved control of blood glucose which can be achieved by standard out-patient treatments, with either multiple daily subcutaneous injections of insulin or oral hypoglycaemic agents, on haemostatic function in both newly diagnosed diabetics without microangiopathy and established diabetics with proven microangiopathy
- iv) the therapeutic effect, if any, of biosynthetic human insulin on haemostatic function in the treatment of established diabetics with proven microangiopathy
- v) the effect of treatment with insulin on haemostatic function in established diabetic patients whose diabetes is normally controlled by treatment with oral hypoglycaemic agents

- vi) the degree of activation of the coagulation pathway in diabetes mellitus as assessed by measuring fibrinopeptide A.

The haemostatic factors which were investigated were as follows:

- i) Factor VIII coagulant activity, (VIII:C)
- ii) Factor VIII von Willebrand related antigen levels, (vWF:Ag)
- iii) Factor VIII von Willebrand related activity as measured by its ristocetin cofactor activity
- iv) Fibrinogen levels, (Fib)
- v) Blood fibrinolytic capacity as assessed by euglobulin clot lysis times, (ECLT), measured on blood samples under the following conditions:
  - a) fasting resting state, no venous occlusion (Resting Euglobulin Clot Lysis Time, R ECLT)
  - and in selected patients
  - b) after a standard period of venous occlusion, (Post Venous occlusion, PV ECLT)
  - c) one hour after administration of insulin by subcutaneous injection, (Post Insulin, PI ECLT).
- vi) Fibrinopeptide A levels, (FPA).

Details of all methods used in the assessment of haemostatic factors are given in Chapter 3.

### 6-3 DETAILS OF NORMAL VOLUNTEER SUBJECTS AND PATIENTS STUDIED

#### 6-3-1 Normal Volunteer Subjects

Special care was taken in attempting to recruit an appropriate control group of age matched healthy normal control subjects. It was the practice of the laboratory at Leeds at the time of these studies to obtain a "normal" plasma pool, on which the standard value of haemostatic function assays was based, from hospital out-patients attending for venesection on whom clinical details were not available. Whilst this is of little importance when testing for the presence of gross deficiency of any one factor, studies of the diabetic patients will be biased if the "normal" populations have minor alterations of coagulation factor levels and activities due to intercurrent illness.

Normal volunteers were recruited from members of the Department of Medicine at Leeds General Infirmary and from blood donors attending the local blood transfusion centre as described in Chapter 2-2. Details and results of this group are given in Table 6-1. There were 15 subjects in the group with 9 males and 6 females. The median

age was 48 (range 24-62 years). None of this group were smokers and none gave any history of significant illness. The median weight index (weight in kilograms divided by the square of the height in metres) was 23.0% (range 19.9-26.5%), where values of roughly between 20% to 25% indicate the ideal range of weight for height (Garrow, 1981). Two subjects were overweight for height by this calculation. For practical reasons, the normal subjects were studied post prandially, as opposed to the fasting state, but were required to rest supine for 30 minutes before blood sampling. Possible dietary effects on haemostatic function are considered in the discussion section of this chapter. Post insulin haemostatic function measurements were not made in this group. These subjects were studied once. Owing to technical difficulties, data is incomplete in 4 cases.

#### 6-3-2 Newly Diagnosed Diabetic Patients with Short Known Duration of Diabetes Mellitus (Type I)

Nine newly diagnosed insulin requiring diabetics, all assumed to be Type I insulin dependent, on the basis of clinical presentation and findings, were recruited as described in Chapter 2-1. Details and results are given in Table 6-2. There were 4 females and 5 males, the median age was 44 years (range 18- 63 years) and median weight index 19.3% (range 16.9-28.9%). Five patients were below ideal weight and 1 above ideal weight for height.

Only one patient (B.F.) was severely ill from diabetes mellitus at diagnosis and he also had mild renal failure and hypertension secondary to a congenital renal abnormality (horseshoe kidney). He was on treatment with atenolol and cyclopentiazide for hypertension. His creatinine clearance was 32 ml/minute per metre<sup>2</sup> (normal range 40 - 90 ml/minute per metre<sup>2</sup>). B.F.'s plasma was noted to be hyperlipaemic at the time of diagnosis but plasma lipids returned to normal with diabetic diet and treatment. Another patient (L.C.) had mild hypertension for which she was receiving treatment with a pinodol/clopamide combined preparation (Viscaldix). No other patient in this group had a history of significant illness. One patient, (H.R.) was a smoker. All these patients were examined at diagnosis by an ophthalmologist using direct fundoscopy and slit lamp examination and none had evidence of diabetic eye disease.

Results of one additional subject (S.H.), who was studied at the time of diagnosis but who had evidence of background retinopathy and diabetic vascular disease in the foot at that time are included in Table 6-2. S.H. was treated with oral agents for one week before being transferred to insulin treatment and was studied whilst receiving insulin.

### 6-3-3 Established Insulin Requiring Diabetic Patients with Microvascular Disease (Type I)

There were 15 patients in this group which consisted of 4 females and 11 males. They were recruited as described in Chapter 2-1 and participated in the double-blind crossover study of treatment with BHI and PPI. Details and results are given in Table 6-3. The median age was 51 years (range 27-67 years). The median known duration of diabetes mellitus was 17 years (range 3-35 years) and the median weight index was 23.4% (range 19.5-30.0%) with 3 patients above the ideal weight for height. This group of patients included 4 subjects initially treated with oral hypoglycaemic agents but transferred to insulin because of failure to control blood glucose levels satisfactorily in 3 cases (G.S., M.W., and P.F.) and because of poor control and painful diabetic neuropathy in the last case (G.B.). The median insulin dose was 44 units, (range 20-80 units).

All patients in this group had diabetic retinopathy on clinical examination confirmed by retinal fluorescein angiography. Details of the eye changes are given in Chapter 9. One patient (D.C.) also had persistent proteinuria assumed to be due to diabetic nephropathy and 2 others had intermittent proteinuria. Only 3 patients had symptomatic neuropathy but all had abnormal peripheral nerve function on electrophysiological testing. One patient (W.H.) had chronic diabetic foot ulcers. Two patients were hypertensive, on treatment, and one patient was on hormonal replacement therapy for menopausal symptoms. One patient (G.B.) gave a history of gout which was quiescent throughout the course of the study and did not require any specific treatment. None of the patients had symptoms of ischaemic heart disease or intermittent claudication but one subject (C.H.) gave a past history of myocardial infarction 21 years prior to entering the study. No clinical notes were available to confirm the diagnosis and the patient has been asymptomatic since then with a current normal electrocardiogram. Electrocardiograms and examination of the peripheral pulses were normal in all the patients. One patient (G.T.) smoked.

### 6-3-4 Established Diabetic Patients Treated with Oral Hypoglycaemic Agents (Type II)

These patients were recruited as described in Chapter 2-1. Details are given in Table 6-4. There were 9 males and 3 females in this group, median age 55.5 (range 38-68). The median weight index was 23.2% (range 18.5-34.8%) with 4 patients overweight assuming a normal range of 20-25%. Four patients (P.W., K.F., A.L., A.H.) had poorly controlled diabetes and were transferred to insulin after the investigations of haemostatic function. The HbA<sub>1c</sub> level of (A.H.) was over 10% but was not given in Table 6-4 as it was measured by an outside laboratory with a different normal range. M.B. also had poorly controlled diabetes mellitus but this was due to diet failure.

Three patients had evidence of atherosclerotic vascular disease; A.L. - on enquiry admitted to symptoms of intermittent claudication in the calf muscles and on examination had diminished peripheral pulses; B.R. - gave a 10 year history of angina and previous myocardial infarctions and was receiving treatment with propranolol, and M.B. - gave a history of hypertension, atrial fibrillation and heart failure diagnosed six years before this study. M.B. was receiving treatment with methyldopa for control of hypertension. M.B. also had bilateral cataracts and two years after the study had developed proliferative retinopathy and suffered a cerebrovascular accident. G.M. was hypertensive and was receiving treatment with methyldopa and cyclopenthiazide 0.25 mg/potassium chloride 600 mg (Navidrex K). K.G. had a history of epilepsy and although fit- free for many years was taking anticonvulsants (phenobarbitone). P.W. had a history of several attacks of pancreatitis (the last attack was ten years before this study) and was presumed to have secondary diabetes mellitus. M.W. had a history of mild hypercalcaemia which investigations suggested was due to primary hyperparathyroidism and her corrected calcium at the time of study was 2.53 m.mol/l, (normal range 2.13-2.43 m.mol/l). M.W. also suffered from depression for which she was receiving treatment with amitryptiline. M.D. gave a history of irritable bowel syndrome and occasionally took codeine phosphate for control of diarrhoea and she was also receiving treatment with oestradiol valerate (Progynova) prescribed by her general practitioner for control of hot flushes, presumed menopausal symptoms. Five patients had evidence of background retinopathy, (P.W., G.M., J.S. M.D. and M.W.) and one patient (J.Q.) had proliferative retinopathy at the time of study. Only one patient, (A.L.) was a smoker.

#### 6-3-5 Summary of Diabetic Patients Studied

Thus a total of 37 diabetic patients were studied, 25 of whom were being treated with insulin and 12 with oral hypoglycaemic agents. The diabetic patients consisted of a) 9 newly diagnosed assumed Type I and an additional patient SH who was initially treated with oral agents for a week before being transferred onto insulin treatment (see Table 6-1), b) 15 established insulin requiring patients, including 12 with classical Type I diabetes mellitus and 3 oral agent failures (who all had evidence of microvascular complications, see Table 6-3) and 12 established diabetics, all treated with oral hypoglycaemic agents, of whom 4 subsequently required treatment with insulin because of poor diabetic control (see Table 6-4). To simplify the discussion, insulin requiring treated patients are referred to as Type I and patients on oral therapy agents as Type II, although C peptide levels are not available to confirm these diagnoses. However, this was not considered important, as the aim of the study was to consider the effect of the type of treatment, insulin versus oral hypoglycaemic agent, rather than the type of diabetes.

The newly diagnosed patients tended to be the thinnest and youngest, the Type II patients the oldest and fattest but there were no significant differences of age and weight index between the diabetic groups. Diabetic control, as measured by HbA<sub>1</sub>C, was however significantly better in the Type II patients, mean 9.6% compared with mean 12.6% in Type I patients,  $p=0.0033$ , (unpaired t test). This reflects the inclusion of newly diagnosed diabetic patients in the Type I group. Fasting blood glucose levels, taken simultaneously with blood for haemostatic factor assays were not significantly different amongst the Type I and Type II patients.

#### 6-4 METHODS AND STATISTICAL ANALYSIS

Methods of measuring the haemostatic function factors are described in Chapter 3. The normal and diabetic population results involved small numbers and it could not be assumed that the data was normally distributed. Therefore the statistical analysis was performed using non-parametric statistical methods. These included the Mann Whitney U test for the comparison of different subgroups, the Wilcoxon rank sign test for testing the effects of improved diabetic control and Spearman's test of rank correlation to examine the influence of variables on haemostatic function. To avoid bias from any sequential trend occurring throughout the study, the results of the double blind crossover study of treatment with biosynthetic human and porcine insulins was examined by doing a Wilcoxon test on the changes in the haemostatic factors which occurred on transfer to each insulin.

The effects of the presence of diabetes mellitus, the influence of other variables such as age, obesity, blood pressure, diabetic control and the effects of a period of improved diabetic control on haemostatic function were examined in the diabetic patients as a whole. Because the patients were a very heterogeneous mixture the analysis was repeated and the results presented separately for the different groups of:

- a) newly diagnosed assumed Type I patients, who had no evidence of vascular disease
- b) established insulin requiring patients with proven microvascular disease (assumed Type I)
- c) the diabetic patients on treatment with oral therapy, of whom 6 had some evidence of microangiopathy.

In addition data from all the insulin treated patients were analyzed separately. A stepwise multilinear linear regression analysis of the effects of multiple variables including age, weight index, blood pressure (systolic and diastolic), duration of diabetes, HbA<sub>1</sub>C, blood glucose, type of treatment for diabetes (whether insulin or tablets), plasma insulin levels, and the presence of diabetic microangiopathy (and whether this was background, proliferative or deteriorating and requiring

photocoagulation treatment) was made. A stepwise multilinear regression of the effects of age, weight index and systolic and diastolic blood pressure was made in the normal control subjects.

#### 6-5 RESULTS OF HAEMOSTATIC FUNCTION TESTS IN NORMAL VOLUNTEERS (CORRELATIONS WITH AGE, FATNESS AND BLOOD PRESSURE)

Haemostatic function tests demonstrated an extremely wide range of normal values. The results of haemostatic function tests on normal volunteers are shown in Table 6-1 and summarized in Table 6-5 and correlations with age, weight index and blood pressure are given in Table 6-6.

##### 6-5-1 Factor VIII Complex

This was measured in 12 subjects of whom 1 female showed abnormally low levels. She had no family history of bleeding disorders. The median factor VIII:C activity was 107.5% (range 36-150%), median vWF:Ag was 119% (range 26-167%) and the median ristocetin cofactor was 89.5%, (range 36-195%). There was no statistical association of age, weight index or blood pressure with factor VIII complex activities.

##### 6-5-2 Fibrinogen Levels

These were measured in 15 subjects and the median value was 1.69 gm/l (range 1.28-2.5 gm/l). In keeping with previous studies, (see discussion) there was a positive association (on Spearman's test) of increasing age with higher levels of fibrinogen ( $p = 0.0106$ ) and of higher weight index values with higher levels of fibrinogen ( $p = 0.0152$ ). Higher blood pressure both systolic ( $p=0.0048$ ), and diastolic ( $p=0.02210$ ), showed a positive correlation with higher levels of fibrinogen. A stepwise multilinear regression analysis suggested that the independent variables were diastolic blood pressure,  $p < 0.05$  and weight index  $p < 0.02$ .

##### 6-5-3 Fibrinopeptide A

FPA was only measured in 11 subjects as the assay was not available at the time of study of some normal controls. The median value was 1.2 pg/ml, (range 0.3-14.5 pg/ml), The upper limit of normal for the assay in the laboratory was 3 pg/ml. Three normal healthy subjects had FPA values over 3 pg/ml, suggesting that activation of thrombin had occurred during venesection. There was no relationship of age or weight index with FPA. On Spearman's test, there was a suggestion of an association of higher blood pressure with higher levels of FPA but  $p$  values for the correlation of systolic and diastolic blood pressure with FPA just failed to reach statistical

significance. However a stepwise multilinear regression analysis of the relationship of FPA and systolic blood pressure did reach statistical significance,  $p < 0.05$ .

#### 6-5-4 Fibrinolytic Activity

The median R ECLT was 101 minutes (range 34 to over 500 minutes) and the median PV ECLT was 70 minutes (range 15 to over 500 minutes). Increasing age was associated with longer PV ECLT ( $p = 0.0058$ ) suggesting that fibrinolytic capacity, the release of plasminogen activator from the vein vessel wall in response to venous occlusion stimuli, is reduced with aging. In 3 subjects no increase in fibrinolytic activity occurred after venous occlusion, suggesting that the stimulus was not optimal. Higher weight index (as has been noted by others, reviewed by Ogston, 1983 [b]) was associated with longer resting ECLT, suggesting that fatness is associated with lower basal fibrinolytic activity, ( $p = 0.0425$ ). A multilinear regression analysis confirmed the association of higher weight index and longer basal R ECLT, and of age and longer PV ECLT. It also revealed that higher systolic blood pressure tended to be associated with shorter PV ECLT, although this just failed to reach statistical significance. High systolic blood pressure may be a sign of stress, which results in high circulating adrenaline levels and hence increased fibrinolytic activity.

In one individual (E.H.) there was no measurable fibrinolytic activity either at rest or after venous occlusion stimuli. The test was repeated with identical results. E.H. had no history of thromboembolic disease and remains in good health! The possible causes of this prolonged ECLT are discussed in section 6-10.

### 6-6 RESULTS OF HAEMOSTATIC FUNCTION IN DIABETIC PATIENTS

Median haemostatic function factors and the results of a Mann Whitney U test comparing all the diabetic subjects with the normal controls are given in Table 6-5. Results of a Spearman's test of correlations of haemostatic factors with various variables in all diabetic patients are shown in Table 6-7 and in the 24 insulin treated patients in Table 6-8.

#### 6-6-1 Factor VIII Related Activities

##### a) Comparison with Normal Volunteers

Factor VIII related activities in all diabetic patients were not significantly different from those of normal controls. The median VIII:C was 100% (range 38 - 300), the median vWF:Ag 97% (range 34 - 320%) and the median ristocetin co-factor 100% (range 29 - 235%). However 4 patients had higher levels of VIII:C, and 5 patients higher levels of ristocetin co-factor than the respective upper limits in normals.



b) Comparison of New versus Established and Insulin Treated versus Oral Agent treated Diabetes Mellitus

Sudivision of diabetics into Type I and Type II revealed that the median Factor VIII:C, vWF:Ag and ristocetin co-factor activities were lower in patients treated with oral agents Type II, compared with insulin treated Type I patients. The median VIII:C in Type II was 90%, versus 110% in Type I diabetics, ( $p=0.0198$ ), the median vWF:Ag in Type II patients was 72.5% versus 113% in Type I diabetics ( $p=0.0016$ ) and the median ristocetin co-factor in Type II diabetics 79% versus 118% in Type I diabetics ( $p=0.0005$ ). The median levels of factor VIII activities in Type II patients were not significantly lower than those of normal controls.

A separate analysis of Type I patients compared with normals was made. Whilst VIII:C and vWF:Ag levels in Type I diabetics (both new and established) were similar to those of normal control subjects, ristocetin co-factor in these insulin treated patients (median 118%) was 32% elevated compared with that of the normal control subjects ( $p = 0.0398$ ) and highest in established Type I patients with microvascular disease (median 126%). It has been suggested that ristocetin co-factor may be a marker of microvascular disease (Boneu et al, 1975). However ristocetin co-factor levels were lowest in Type II patients of whom half had microvascular disease, 5 with background retinopathy and 1 with proliferative retinopathy.

6-6-2 Results of Spearman's Analysis of Correlation of Factor VIII with Various Variables

a) All Diabetic Patients

Association of diabetic control with factor VIII activities was suggested by statistical correlations (Spearman's test) of factor VIII with HbA<sub>1</sub>C and blood glucose levels. In the diabetic patients as a whole, poor diabetic control as measured by HbA<sub>1</sub>C levels was associated with higher levels of factor VIII:C ( $p = 0.0122$ ) but not with the other Factor VIII activities, (see Figure 6-1).

b) Patients Treated with Insulin

In Type I insulin treated patients diabetic control, as assessed by HbA<sub>1</sub>C, was associated with vWF:Ag ( $p = 0.0165$ ), see Figure 6-2 but less significantly with VIII:C levels ( $p = 0.0591$ ). There was no association between HbA<sub>1</sub>C% and ristocetin co-factor activity (see Table 6-8).

However in Type I patients, the fasting blood glucose level was correlated with ristocetin cofactor activity ( $p = 0.00376$ ), see Figure 6-3. As acute diabetic decompensation has been reported to result in high levels of Factor VIII (Paton 1981),

separate analysis of the association of diabetic control and Factor VIII activities was made using Spearman's test in new and established Type I diabetic patients. In established Type I patients there was a strong association of VIII:C and HbA<sub>1</sub>C,  $p = 0.0064$  and also between vWf:Ag and HbA<sub>1</sub>C,  $p = 0.0058$ . In newly diagnosed Type I patients, there was also a correlation between HbA<sub>1</sub>C and vWF:Ag ( $p < 0.05$ ).

#### c) Patients Treated with Oral Agents

In the Type II diabetic patients, there was no association of diabetic control as assessed by HbA<sub>1</sub>C and Factor VIII:C or vWF:Ag but an association of HbA<sub>1</sub>C and ristocetin co-factor was noted, with poor diabetic control being associated with higher levels of ristocetin co-factor ( $p = 0.0217$ ). In these Type II patients it was also noted that fatter patients had higher levels of vWF:Ag ( $p = 0.0307$ ).

### 6-6-3 Stepwise Multilinear Regression Analysis of the Association of Factor VIII and various Variables

#### a) VIII:C

A stepwise multilinear regression analysis of the data from all the diabetic patients revealed that independent predictors of high levels of VIII:C were poorly controlled diabetes as measured by both blood glucose and HbA<sub>1</sub>C, long duration of diabetes and deteriorating retinopathy,  $p < 0.05$  in each instance.

#### b) VIII:Ag

The multilinear regression analysis revealed that high VIII:Ag levels were also associated with long duration of diabetes and the presence of deteriorating retinopathy,  $p < 0.05$ , in each instance, and with poorly controlled diabetes as measured by HbA<sub>1</sub>C levels,  $p < 0.001$ . There was no independent association with blood glucose levels.

#### c) Ristocetin Cofactor

The independent predictors of high levels of ristocetin cofactor were high blood glucose levels,  $p < 0.01$ , high diastolic blood pressure,  $p < 0.05$ , and treatment with insulin as oppose to oral agents  $p < 0.001$ . There was no association of HbA<sub>1</sub>C and ristocetin cofactor levels.

### 6-6-4 Fibrinogen Levels

Fibrinogen levels were 41% higher in the diabetic patients, median 2.38 gm/l compared with normals, median 1.69 gm/l, ( $p=0.0005$ ). Fourteen patients had fibrinogen levels higher than the upper limit of the normal control subjects. Fibrinogen levels were significantly higher in all diabetic groups compared to normal control subjects,

established Type I median 2.32 gm/l, Type II, median 2.34 gm/l and newly diagnosed Type I median, 2.7 gm/l.

Unlike the normal control subjects, there was no association of fibrinogen levels and weight index or age; but poor diabetic control as measured by HbA<sub>1c</sub> levels was associated with higher levels of fibrinogen on both Spearman's test,  $p = 0.0347$  (see Figure 6-3) and on multilinear regression of all variables,  $p < 0.05$ . This association of diabetic control with fibrinogen levels appeared to be stronger in insulin treated Type I patients,  $p < 0.01$  for both new and established patients but was not noted in the Type II patients when the data was analyzed separately. Stepwise regression revealed that independent variables predicting higher levels of fibrinogen were poorly controlled diabetes as measured by HbA<sub>1c</sub>,  $p < 0.001$ , high diastolic blood pressure,  $p < 0.02$ , the presence of deteriorating retinopathy,  $p < 0.01$  and treatment with oral agents as oppose to insulin,  $p < 0.02$ .

#### 6-6-5 Fibrinopeptide A

This was measured in the 15 established diabetic subjects with retinopathy. Four serial measurements were taken on each patient (see Table 6-12). The FPA measurements taken at the first attendance contained 9 abnormally high results which may be artifactual. A batch of anticoagulant was employed which gave unpredictable results. As the samples were stored and then measured in batch assays the effects of the faulty anticoagulant were not detected immediately. All the samples for FPA measurement taken at the last visit were collected into reliable anticoagulant and only 3 abnormal results were obtained. Data from the last visit was used to compare diabetic and normal control subjects in whom the same anticoagulant and methods were used. Diabetic patient FPA levels, median 2.19 pg/ml (range 0.3-60 pg/ml) were not significantly different from those of normal subjects. There was no association of FPA and measures of diabetic control<sup>1</sup>. In the diabetic patients FPA showed a relation to blood pressure, being positively related to both the systolic ( $p = 0.038$ ) and diastolic blood pressures ( $p = 0.0357$ ).

#### 6-6-6 Fibrinolytic Activity

##### a) Comparison with Normals

Fibrinolytic activity as assessed by resting and PV ECLT was not significantly different in diabetic subjects and all results fell within the range defined for the normal control subjects. There were no significant differences in fibrinolytic capacity as assessed by ECLTs between the diabetic patient groups. (PV ECLTs from the new Type I patients

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<sup>1</sup> Data from the final visit was used in the analysis of association of FPA and Diabetic control etc., not the data presented in Table 6-3

were not included in the analysis, see section 6-7). Post insulin ECLTs were examined in the established Type I patients. One hour after injection of insulin there was a shortening of ECLT from a median of 111 to 86 minutes indicating an increase of blood fibrinolytic activity. At the time of PI ECLT measurement the median blood glucose was 15 mmol/l, range 11.2 - 27.3 mmol/l and thus the changes in fibrinolytic activity would not appear to be mediated by hypoglycaemia.

#### b) Spearman's Analysis of Correlations with various Variables

In the diabetic patients as a whole, Spearman's test did not reveal any association of diabetic control (as assessed by HbA<sub>1c</sub> and blood glucose levels) with resting ECLT but it was noted that diastolic blood pressure showed a positive association with R ECLT,  $p = 0.0459$ . In insulin treated patients, diabetic control, as assessed by HbA<sub>1c</sub> levels was significantly related to resting basal ECLTs ( $p = 0.0316$ ) but the relationship with diastolic blood pressure failed to reach statistical significance. In Type II patients, Spearman's test did not reveal any association of diabetic control or blood pressure and fibrinolytic activity, but fatter patients had less basal fibrinolytic activity as indicated by a positive association with resting ECLT,  $p=0.0307$ .

#### c) Stepwise Multilinear Regression

A stepwise multilinear regression of all variables on all patients revealed that the independent variables associated with less basal fibrinolytic activity in blood were high diastolic blood pressure  $p < 0.05$  and poor diabetic control as measured by HbA<sub>1c</sub>  $p < 0.05$ .

### 6-7 RESULTS OF THE EFFECTS OF A PERIOD OF IMPROVED DIABETIC CONTROL ON HAEMOSTATIC FUNCTION

#### 6-7-1 All Patients

The effects of a short period of improved diabetic control was examined in 31 of the patients, 14 established Type I who participated in the BHI - PPI study, 10 newly diagnosed patients including SH and 6 Type II established diabetic patients. The protocol for studies of improved control is given in chapter 2-3. The newly diagnosed patients and SH were examined at diagnosis and after 14 and 40 days of good control, and the others at entry to the study and after 90 days. Results on are given in Table 6-9. Improvement in diabetic control was confirmed by a fall in the median HbA<sub>1c</sub>% from 12.1 to 9.8% ( $p < 0.0001$ ) and of the median blood glucose from 10.8 to 8.1 mmol/l ( $p < 0.05$ ). The significant effects on haemostatic function factors were a 13.0% fall in the median level of vWF:Ag, ( $p=0.0065$ ), whilst ristocetin cofactor activity showed a statistical increase of 3.5%, ( $p=0.0268$ ) but such a small change is of dubious biological significance.

### 6-7-2 Newly Diagnosed Type I Patients

The haemostatic investigations were carried out in 9 patients, a) at diagnosis, before commencing treatment with insulin, b) at 14 days and c) 40 days after starting treatment with insulin. Post venous occlusion ECLT and FPA measurements were not made in this group. Results are given in Table 6-10.

Improvement in the diabetic control in the newly diagnosed diabetic patients over the ensuing 40 days was confirmed by a steady fall in HbA<sub>1</sub>C levels ( $p < 0.01$ ) and significant sustained reduction in blood glucose levels ( $p < 0.01$ ). Fibrinogen levels remained elevated compared with normal individuals at both day 14 and day 40.

The resting ECLT was significantly prolonged at day 14 ( $p < 0.05$ ) compared with day of diagnosis. Changes in ECLTs have to be interpreted with caution in these 9 patients because venous occlusion tests were performed at diagnosis using both a prolonged 20 minute period of venous occlusion and a higher occlusion pressure than in subsequent experiments. The patients found this procedure very uncomfortable and the protocol was altered. In its original form the test is likely to have produced maximum release and secondary depletion of plasminogen activator from the venous wall. The apparent prolongation of resting ECLTs at day 14 may be partly explained by incomplete recovery from the first venous occlusion test. Factor VIII:C and vWF:Ag levels both fell but only the changes in the vWF:Ag levels were statistically significant ( $p < 0.05$ ). Ristocetin cofactor levels were not significantly altered over the 40 day period.

### 6-7-3 Established Insulin Treated Diabetic Patients with Microvascular Disease

Haemostatic function was examined on 4 occasions: at entry to the study, after a 90 day period of attempted improved diabetic control and after 90 days treatment with each of purified porcine and human insulins (for protocol see 2-4).

Results of Improved Diabetic Control are given in Table 6-11. Following the 90 day period of improved diabetic control, which formed the run-in period to the double-blind crossover BHI-PPI study, median HbA<sub>1</sub>C levels showed a small but significant fall from 10.7% to 10.1%, ( $p < 0.05$ ). Fasting blood glucose levels were not significantly different. There was a small fall in the median resting ECLT from 111 to 85 minutes indicating an increase in resting blood fibrinolytic activity ( $p = 0.0393$ ) and the median PV ECLT also fell from 62 to 29 minutes but this change was not significant. The PI ECLT values were unchanged. Fibrinogen levels remained elevated compared with the normal control subjects. The median factor VIII:C, vWF:Ag and ristocetin cofactor levels all fell but the changes were not statistically significant. FPA levels fell from a median value of 4.6 to 3.4 pg/ml ( $p < 0.05$ ) but these changes represented part of an overall trend which continued throughout the double blind study

of PPI and BHI and the changes were probably due to improved methods of collecting blood samples for this assay, (see Table 6-12)

#### 6-8 COMPARISON OF HAEMOSTATIC FUNCTION ON TREATMENT WITH PURIFIED PORCINE AND BIOSYNTHETIC HUMAN INSULINS

The protocol in chapter 2-4 was followed. Data from all 15 established type I patients was analyzed. Results are shown in Table 6-13. During treatment with purified porcine and biosynthetic human insulins in the double blind phase of the study, there was no further improvement in diabetic control. There were no detectable differences of any of the parameters of haemostatic function on either insulin.

#### 6-9 THE EFFECTS OF TREATMENT WITH INSULIN ON HAEMOSTATIC FUNCTION IN DIABETIC PATIENTS NORMALLY TREATED WITH ORAL HYPOGLYCAEMIC AGENTS

The effects of a 90 day period of treatment with purified porcine or biosynthetic human insulins was examined in 3 patients normally well controlled on oral hypoglycaemic agents (see Table 6-14 for results). The period of insulin treatment was preceded by a 90 day period of attempted improved control of blood glucose levels through advice and education and adjusting oral hypoglycaemic therapy as necessary. Haemostatic function tests were measured at the beginning of the study, after the run-in period on oral hypoglycaemic drugs and after 90 days treatment with insulin.

Diabetic control as assessed by  $HbA_{1C}$  and fasting whole blood glucose levels improved during the run-in period but deteriorated on transfer to treatment with insulin in two patients. There were no consistent changes in the factor VIII related activities, fibrinogen levels or resting ECLTs on treatment with insulin.

#### 6-10 DISCUSSION

One problem with haemostatic function factors studies is the interpretation of laboratory tests which may not reflect the in-vivo interactions and dynamic changes of the haemostatic mechanism. Recent data suggests that the vascular endothelium is actively involved in the synthesis of factors that prevent and limit blood coagulation, as well as others that lead to the activation of platelets and the plasma clotting system (as reviewed by Porta et al, 1987). Descriptions of levels of plasma clotting factors may not accurately reflect local vascular interactions, and as haemostatic function factors are normally present in great excess, the significance of mild elevation of any one factor has to be interpreted with caution. Proponents for the theory, that the excess of haemostatic factors in diabetic blood results in a hypercoagulable state, argue (on the basis of Virchow's classical triad) that disturbances of haemostatic variables, in association with abnormalities of endothelial function and disturbances of blood flow

and microvascular reflexes, might convert a temporary obstruction of capillary flow into a pathological capillary closure by promoting thrombosis (see Chan et al, 1982; Haitas et al, 1984[a]; Stenberg et al, 1984). The combined effects of these disturbances may explain the susceptibility of diabetic patients to vascular disease.

Reliable and accurate haemostatic assays have only been available relatively recently. During the 1970's, general principles of biological standardization were applied to the measurement of haemostatic components and stable reference standards for the most important factors were established. However, virtually all collaborative studies carried out by the National Institute for Biological Standards and Control in the field of Haemostasis have found striking discrepancies between different laboratories potency estimates on the same samples (Barrowcliffe & Kirkwood, 1981). A particular problem has been found with regard to estimates of Factor VIII (Kirkwood & Barrowcliffe, 1978). Therefore it was felt important to establish ranges for the coagulation factors in normal healthy volunteers.

#### 6-10-1 Normal Ranges for the Haemostatic Factors under Study and the Relationship of Factors with Age, Obesity and Blood Pressure

##### a) Normal Fibrinogen

For practical reasons, only small numbers of normal control subjects could be examined. However the findings are consistent with those of much larger series, supporting the validity of these studies. In normal healthy adults, association of higher fibrinogen levels with increasing age and fatness was found. Similar findings are reported by a) Bailesien et al, (1985), b) Meade et al, (1979), and c) Korsan-Bengtson et al, (1972), who all examined large series containing several thousand (a and b) and several hundred patients (c). The reasons why fibrinogen levels alter with age are not, as yet, understood. In the all three studies mentioned, fibrinogen levels were also higher in cigarette smokers. In the authors studies, none of the normal controls smoked although 2 of the patients did. However the author found a strong association of fibrinogen levels with blood pressure, a finding which was also noted in the early study by Hamilton et al (1974), who showed a positive of correlation fibrinogen levels with blood pressure, with older patients being more hypertensive.

##### b) Normal Fibrinolytic Activity

In the current studies fibrinolytic activity, (as measured by ECLT) was less in older and fatter control subjects. Reduced fibrinolytic activity in the obese, is a well-recognized but poorly understood phenomenon (as reviewed by Ogston, 1983[a]). Most studies have not found any association with age (as reviewed by Ogston, 1983 [b]) but Meade et al (1979), reported a decrease in blood fibrinolytic activity on comparing 20-

25 year olds with 50- 55 year olds. Again, the reasons for these age and obesity related changes of fibrinolytic activity remain uncertain.

In the author's studies, there was one normal individual, (a 62 year old male, weight index 26%, with a normal plasma fibrinogen), whose ECLT did not show any activity, either in the basal sample or after venous occlusion testing, indicating marked hypofibrinolysis. Abnormal plasminogen activator and faulty generation of plasmin has been linked with susceptibility to recurrent deep venous thrombosis (see Davidson & Walker, 1981), but there was no history of DVT in this healthy control. Further investigations into the cause of the abnormally prolonged ECLT were not pursued.

Venous occlusion stimulates release of plasminogen activator from vein wall, and is used as a measure of fibrinolytic capacity. The original test, introduced by Robertson (1972), was uncomfortable and poorly tolerated by subjects. A modified venous occlusion test, using a shorter period of occlusion and lower pressure than that first described, was employed by the author. In 2 of the normal control subjects, there was no increase in fibrinolytic activity on venous occlusion, indicating that the occlusion stimulus was not optimum. However, more prolonged venous occlusion test was undesirable, because of the plan to repeat the studies in the diabetic test group. After prolonged stimulation by venous occlusion, the fibrinolytic capacity of arm veins is exhausted and does not return to normal for over fourteen days (Keber and Stegnar, 1982).

#### c) Normal Factor VIII Activity

The normal ranges of Factor VIII activities, defined by the author, were essentially a comparison of the selected normal controls' plasma with the pooled plasma obtained from hospital outpatients which was used to define the 100% activity values. In healthy individuals, raised levels of factor VIII:C and von Willebrand factor have been reported in association with increasing age (Bailesien et al, 1985; Aillaud et al, 1986). In the normal control subjects studied by the author, no associations of factor VIII:C or von Willebrand factor, as measured by its antigen levels or associated ristocetin cofactor activity, were found with age or obesity.

#### d) Normal Fibrinopeptide A

FPA levels were only measured in ten normal control subjects. Abnormally high levels were found in 3 healthy subjects, suggesting that there had been activation of thrombin during venesection. No particular associations of obesity and age were found with FPA, though there was a trend which just failed to reach statistical significance of higher levels of FPA with higher levels of blood pressure.



6-10-2 The Effect of the Presence of Diabetes Mellitus on Haemostatic Function and the Relationship with Age, Obesity, Blood Pressure Diabetic Control, Treatment and Presence of Diabetic Complications

a) Fibrinogen

Fibrinogen levels were elevated in diabetic patients studied, an expected finding, (as reviewed in chapter 1-6). Multilinear regression analysis revealed that diabetic control, as measured by  $HbA_{1C}$ , was the most significant independent predictor of fibrinogen levels. Other independent variables were high blood pressure, the presence of deteriorating retinopathy and treatment with insulin as oppose to oral agents. Unlike normal subjects, no association with weight index or age was found. Fibrinogen levels were also found to be highest in diabetic patients with proliferative retinopathy in an earlier study by Lowe et al (1980). These strong associations, of fibrinogen with current diabetic control and severity of vascular disease, may reflect the role of fibrinogen as an acute phase reactant protein.

b) The Effects of Improving Diabetic Control on Fibrinogen Levels

As fibrinogen has a short plasma half-life of three to five days (reviewed by Reeve & Franks, 1974), improvement of diabetic control should result in a prompt fall of fibrinogen levels, if diabetic control is a significant determinant. In this study, no significant change of fibrinogen levels occurred on improving diabetic control. In an earlier study Tymms (1985) found the reverse - treatment with subcutaneous continuous insulin infusion for 10 days, to achieve near normalization of diabetic control, resulted in a marked increase of fibrinogen levels, in Type II patients previously poorly controlled on oral agents. There are several possible explanations for this apparent anomaly. First, insulin may stimulate fibrinogen production (this has been demonstrated in cultured chick embryos, see Ogston 1983, [d]) and thus introduction or intensification of insulin treatment may potentially increase fibrinogen production. However, the role of insulin in the physiological regulation of fibrinogen levels in humans is not known. Secondly, glycosylation of fibrinogen, as occurs in diabetes mellitus, has been reported to increase resistance to breakdown by plasmin and hence to slow removal of fibrinogen from the circulation (Brownlee et al, 1983). But no alterations of glycosylated fibrinogen function, as measured by thrombin clotting time, plasmin digestion or heat stability were noted in an earlier study by McVerry et al (1981) and the findings of Brownlee's group require further confirmation. In fact, other studies have suggested that fibrinogen survival is reduced in poorly controlled diabetes mellitus, and that this is rapidly reversible with improved control of blood glucose levels (Jones and Peterson, 1979). Suggested stimuli to fibrinogen production, also include trauma and tissue inflammation (effects perhaps mediated by leucocyte related factors, prostaglandins, growth hormone, ACTH, thrombin). In uncontrolled

diabetes mellitus, associated hormonal and prostaglandin disturbances or vascular injury might provide the stimuli for increased fibrinogen production. Continuing over-production in spite of decreased consumption seems the most likely explanation of the persistent hyperfibrinogenaemia seen in the early days of improved blood glucose control. Fibrinogen levels have been shown to remain elevated for some months after commencing treatment in newly diagnosed diabetic patients before finally returning to normal (Barnes 1981). A possible explanation is, that cytotoxic damage to the vascular endothelium resulting from the acute diabetic state heals slowly, and hyperfibrinogenaemia is again part of the subsequent acute phase protein reaction.

### c) Fibrinolytic Activity in Diabetes Mellitus

Fibrinolytic activity in the diabetic patients of the author's series was not significantly different from that of normal control subjects. Data on fibrinolytic activity after venous occlusion is incomplete, as the initial 20 minute venous occlusion test (Robertson et al, 1972), was poorly tolerated by subjects and as mentioned above, it was modified during the course of study. In the diabetic patients, high diastolic blood pressure was an independent predictor of lower basal blood fibrinolytic activity. Associations of low fibrinolytic capacity and high blood pressure, as found by the author, are not well recognized. Blood pressure is often higher in the obese and elderly and diabetic in whom fibrinolytic capacity is reported as being reduced.

The other independent predictor for lower levels of basal blood fibrinolytic activity was poor diabetic control as measured by HbA<sub>1c</sub> levels. On Spearman's test of correlation, an association of HbA<sub>1c</sub> and fibrinolytic activity was noted in insulin treated diabetic patients, but not in patients treated with oral therapy agents. It has been suggested that sulphonyurea and biguanide agents have a direct fibrinolytic effect (Fearnley et al, 1960; Banerjee et al, 1975), but in this series, treatment with oral therapy agents did not emerge as a independent determinant of fibrinolytic activity on the multilinear regression analysis. Other differences between patients may account for this discrepancy.

The many reports of basal resting fibrinolytic activity in diabetes mellitus give conflicting results, but series examining fibrinolytic capacity (on stimulation testing with adrenaline, venous occlusion and exercise) generally agree that activity is reduced (see chapter 1, section 6-7). This suggests that production of venous plasminogen activator is reduced in diabetes mellitus. As plasminogen activator is released from vascular endothelial cells, impairment of this response may be an indication of impaired endothelial cell function. However, it has not been established whether decreased fibrinolytic capacity is due to reduced synthesis of plasminogen activator, impaired release from vascular endothelial stores, increased production of inhibitors or a combination of these mechanisms. In diabetes mellitus, plasminogen activator

secretion may be first enhanced through a variety of stimuli, including associated hormonal disturbances, treatment with insulin and drugs and, in theory, this could result in secondary depletion of venous plasminogen activator and lesser fibrinolytic capacity. However, interpretation of stimulation tests is limited, as their physiological significance is not known.

#### d) The Effects of Improved Diabetic Control on Fibrinolytic Activity

In the newly diagnosed diabetic patients studied by the author, there was a reduction of fibrinolytic activity of blood 14 days after introduction of treatment with insulin. In these patients, the prolonged venous occlusion test (Robertson et al, 1972) was used at diagnosis. This may have resulted in depletion of venous plasminogen activator stores. Improved blood glucose control in established Type I patients, in whom a lesser venous occlusion stimulus was applied and in whom there was a longer interval before the second test, allowing full recovery of venous plasminogen activator stores, resulted in an apparent increase in basal blood fibrinolytic activity. Post venous occlusion fibrinolytic activity was unchanged. The association of basal fibrinolytic activity with HbA<sub>1c</sub> and the increase in basal fibrinolytic activity after a period of improved diabetic control in established Type I patients, suggests that better diabetic control might be associated with greater blood fibrinolytic activity.

#### e) The Effects of Insulin on Fibrinolytic Activity

Insulin has also been reported to result in shortening of blood fibrinolytic activity (Fearnley et al, 1959). This effect has previously been attributed to concurrent hypoglycaemia (Hedlin, 1973). In the author's study, fibrinolytic activity was increased one hour after injection of insulin, despite absence of hypoglycaemia and thus any adrenaline mediated effect. However, the post- insulin increase in fibrinolytic activity found, could still be unrelated to insulin injection. The post-insulin ECLT measurements were made after breakfast and patients were given tea containing caffeine. Caffeine is known to increase fibrinolytic activity (Samarrae & Truswell, 1977). Dietary factors may also have influenced the comparison of normal subjects and diabetic patients. For practical reasons, diabetic patients were examined in the morning, in the fasting resting state. The control subjects, whilst rested before venesection, were not fasted. In view of influences of caffeine containing beverages and diurnal variations on fibrinolytic activity (Fearnley et al, 1957), ideally normal control subjects and diabetic patients should be examined under identical conditions.

#### f) Factor VIII Activities in Diabetic Patients

Overall, Factor VIII activities were not significantly different from those of normal subjects but a strong association of diabetic control as measured by HbA<sub>1c</sub> and blood

glucose and factor VIII:C and vWF:AG levels was noted. Other independent predictors of high levels of Factor VIII:C and vWF:Ag activities were long duration of diabetes mellitus and the presence of deteriorating retinopathy. Independent predictors of the ristocetin cofactor were high diastolic blood pressure and blood glucose levels.

HbA<sub>1</sub>C, in the assay method used in this thesis, measures changes in erythrocytes which respond gradually over the course of days to changes in the average blood glucose and gives no indication of hour to hour fluctuation in blood glucose levels. Von Willebrand factor, as measured by ristocetin cofactor activity was not correlated with HbA<sub>1</sub>C levels but showed a positive correlation with blood glucose at the time of testing, suggesting that functional activity of von Willebrand factor responds more immediately to changes in blood glucose. As the main storage site of von Willebrand factor is the endothelium, particular interest in the role of von Willebrand Factor as a marker of endothelial damage has been generated. Perfusion of endothelium with elevated glucose concentrations in vitro, has been shown to result in release of von Willebrand factor from human umbilical vein (Mordes et al, 1983), suggesting that direct effects of glucose on the vascular endothelium may lead to the release of von Willebrand factor.

#### g) The Effect of Treatment, Insulin versus Oral Agents on Factor VIII Activities

It was noted that factor VIII:C, vWF:Ag and ristocetin cofactor levels were lower in patients treated with oral agents than in patients on insulin. In the multilinear regression analysis, of the influence of variables on factor VIII, insulin treatment was an independent predictor of higher levels of ristocetin cofactor measurements of von Willebrand factor. Diabetic control, as measured by HbA<sub>1</sub>C, was slightly better in the oral therapy group and lower levels of Factor VIII activities may also be partly due to better diabetic control. On stepwise multilinear regression analysis, plasma insulin levels did not emerge as a significant determinant of haemostatic factor levels and the transfer of 3 patients, normally well controlled on oral therapy agents, onto insulin was not associated with any dramatic changes in coagulation factors but insulin requirements were low. Small and colleagues (1986), in a study of the effects of insulin treatment in 20 patients (who had poorly controlled diabetes mellitus on maximal treatment with oral agents) similarly did not note any change in Factor VIII:C or Factor VIII:AG levels. Whether differences in coagulation factors, in the author's study, between patients on oral antidiabetic drugs and patients on insulin, were due to treatment or intrinsic differences in the patients is debatable. Proportionally more of the patients treated with insulin had proven diabetic microangiopathy, whose presence in many studies has been associated with higher factor VIII levels.

### 6-10 3 Fibrinopeptide-A in Diabetes Mellitus

Direct evidence for activation of the haemostatic system as assessed by measurements of FPA was lacking in the present studies of diabetes mellitus but the numbers involved were small. FPA levels were studied sequentially in 15 established diabetic patients and levels fell throughout the course of the studies. During this time, blood sampling technique became defter, and the anticoagulant into which the blood sample was collected was changed as the first batch was unreliable. Subjects in whom venepuncture was difficult had the highest levels of FPA and the usefulness of basal FPA measurements is perhaps limited, as any raised level is suspicious of poor venesection technique. Unless some objective measurement of excellence of venesection technique and speed of blood flow is made, raised levels of FPA levels must be interpreted with caution. However, an association of higher blood pressure with higher FPA levels was noted in both normal and diabetic patients in this study. Others have found marginally higher levels of FPA in diabetic patients but do not report on the association with blood pressure (Borsey et al, 1984; Small et al, 1987). In the latter study, levels of FPA, studied sequentially for 3 months, also fell. During Small et al's study, diabetic control did not improve and the fall in FPA levels was attributed to treatment with insulin in patients normally controlled on oral agents.

### 6-10-4 Effects of Treatment with Biosynthetic Human Insulin

Treatment with biosynthetic human insulin was not associated with any significant differences in haemostatic function factors compared with treatment with purified porcine insulin. In view of the questionable effect of insulin on haemostatic function factors, the negative result of the study of biosynthetic human insulin on haemostatic factors is hardly surprising. Given the limited understanding of the haemostatic system, empirical studies such as this have a role in the assessment of a new treatment with regard to any potentially unexpected beneficial or deleterious effects.

### 6-10-5 Conclusions

Haemostatic factors have been extensively studied in diabetes mellitus, but, as mentioned at the beginning of this chapter, there has been confusion as to the importance of metabolic control, treatment, and vascular disease on levels of haemostatic variables. This confusion may reflect on a lack of inter-laboratory standardization of assays of haemostatic factors in early studies, as well as on the marked heterogeneity of diabetic patients.

Descriptive studies of the levels of haemostatic factors in diabetes mellitus (such as this author's), highlight the important predictors of levels of circulating factors. They do not increase our fundamental understanding of the in-vivo effects of these changes in

diabetes mellitus. Simple associations are not proof of a causal relationship with the development of vascular damage. Future progress in the study of haemostasis and diabetes mellitus, requires knowledge of factors controlling the production of haemostatic factors and of the interaction of factors with the vascular endothelium. No direct evidence for a hypercoagulable state in association with diabetes has been found and the case for the presence of significant hypercoagulability in diabetes mellitus remains non-proven.

**APPENDIX****TABLES TO CHAPTER 6**

**TABLE 6 -- 1**  
**Normal Control Subject Details and Haemostatic Function Results**

Subject	R.B.	E.M.	D.P.	J.P.	P.C.	P.O'L.	M.Le	R.D.	R.H.	C.W.	G.O'L.	S.J.	R.C.	M.T.	M.L.A.	Lowest	Highest	Median
Sex (F/M)	M	M	M	M	M	M	M	M	M	M	F	F	F	F	F			
Age (Years)	24	26	28	31	47	48	48	50	62	43	44	48	49	58	48	24	62	48
Weight Index (Kg/m <sup>2</sup> )	22.0	24.0	23.0	22.0	23.0	24.8	20.0	24.2	26.0	20.9	19.9	23.1	21.7	26.5	23.6	19.9	26.5	23.0
Blood Pressure	120/80	105/75	130/80	100/60	130/90	110/60	120/80	120/80	140/100	105/75	120/80	120/70	120/80	150/100	140/90	100/60	150/100	120/80
Factor VIII:C (%)	115	NA	NA	NA	100	70	120	150	130	95	56	115	36*	68	130	36	150	107.5
Factor vWF:AG (%)	65	NA	NA	NA	165	81	131	124	151	117	26	72	121	43	167	26	167	119
Ristocetin Co factor Activity (%)	89	NA	NA	NA	90	82	115	115	70	98	36	82	110	46	195	36	195	89.5
Fibrinogen (gm/l)	1.64	1.69	1.55	1.45	1.87	1.42	1.68	1.93	2.48	1.51	1.28	2.26	1.83	2.50	1.89	1.28	2.50	1.69
Fibrinopeptide A (pg/ml)	NA	NA	NA	NA	6.3	1.8	0.3	0.3	2.2	1.1	4.7	1.2	1.1	14.5	0.6	0.3	14.5	1.2
Resting Euglobulin Clot Lysis Time (Minutes)	92	230	345	182	34	135	101	345	>500*	41	50	36	75	128	39	34	>500	101
Post Venous Occlusion Euglobulin Clot Lysis Time (Minutes)	NA	NA	NA	NA	15	103	96	364	>500*	29	18	21	44	101	NA	15	>500	70



TABLE 6 - 2

Newly Diagnosed Diabetic Patient Details and Haemostatic Function Results

Subject	R.K.	P.L.	A.B.	B.F.	H.R.	C.R.	S.B.	L.C.	M.N.	Lowest	Highest	Median	Additional Patient S.H.
Sex (F/M)	M	M	M	M	M	F	F	F	F				M
Age (Years)	26	31	44	44	55	18	25	55	63	18	63	44	60
Weight Index (Kg/m <sup>2</sup> )	20.7	16.8	23.9	17.1	19.3	16.9	17.5	28.9	20.1	16.8	28.9	19.3	19.83
Blood Pressure	120/70	120/60	138/74	120/70	135/80	110/55	110/64	140/90	98/50	98/50	140/90	120/70	110/68
HbA <sub>1c</sub> (%)	11.2	13.1	14.2	24.5	16.8	14.5	13.1	15.7	15.1	11.2	24.5	14.5	13.7
Whole Blood Glucose (mmol/l)	10.8	8.2	10.6	77.0	10.9	12.3	11.9	13.0	16.4	8.2	77.0	11.9	13.2
Factor VIII:C (%)	92	110	170	300	110	76	98	80	125	76	300	110	100
Factor vWF:AG (%)	90	89	150	320	121	77	92	111	180	77	320	111	130
Ristocetin Co factor Activity (%)	100	98	190	NA	107	84	94	145	185	84	190	103.5	135
Fibrinogen (gm/l)	1.86	2.05	2.76	3.50	3.24	1.76	1.90	3.36	2.70	1.76	3.50	2.70	NA
Resting Euglobulin Clot Lysis Time (Minutes)	270	195	80	260	330	160	125	185	95	80	330	185	NA
Serum Insulin	8.2	10.5	15.7	11.6	4.5	NA	3.8	19.1	5.2				10.3

TABLE 6 - 3  
Established Insulin Treated Diabetic Patient Details and Haemostatic Function Results

Subject	G.S.	G.T.	R.K.	G.B.	P.F.	D.C.	A.E.	C.H.	R.G.	M.W.	P.P.	W.M.	W.H.	M.T.	D.D.	Lowest	Highest	Median
Sex (F/M)	M	M	M	M	M	F	F	M	M	F	F	M	M	M	M			
Age (Years)	48	40	67	62	57	40	55	51	31	50	51	62	47	27	52	27	67	51
Weight Index (kg/m <sup>2</sup> )	24.3	23.4	21.7	30.0	25.8	28.0	22.2	24.4	21.2	25.8	19.5	22.7	25.0	22.9	22.0	19.5	30.0	23.4
Blood Pressure	150/90	120/80	180/90	180/80	160/100	150/90	140/70	130/90	120/80	140/80	150/90	130/70	140/80	90/50	140/70	90/50	180/100	140/80
Duration of Diabetes (Years)	10	28	14	3	11	20	18	17	15	16	21	35	20	22.5	29	3	35	17
Insulin Dose (Units)	32	48	20	28	44	77	20	70	32	40	52	47	80	50	40	20	80	44
Retinopathy (Stable/Deteriorating)	S	S	D	D	S	D	S	S	S	S	S	S	S	S	S			
HbA <sub>1c</sub> (%)	9.2	10.8	10.4	8.4	11.0	12.6	10.6	10.4	11.8	13.8	13.5	8.5	11.6	12.2	9.5	8.4	13.8	10.6
Fasting Whole Blood Glucose (mmol/l)	6.6	13.8	6.1	8.6	9.7	21.2	10.8	13.8	4.1	7.0	13.3	12.5	8.2	22.2	9.2	4.1	22.2	9.2
Fasting Insulin (uU/ml)	20.0	12.0	7.0	22.0	200.0	11.0	5.0	18.0	6.0	12.0	5.0	13.0	14.0	4.0	8.0	4.0	200.0	12.0
Factor VIII:C (%)	54	84	130	57	48	230	110	72	160	100	140	150	115	150	82	48	230	110
Factor vWF:AG (%)	60	81	204	55	52	168	104	71	115	162	133	152	115	191	82	52	204	115
Ristocetin Co factor Activity (%)	108	126	176	80	96	235	155	175	118	80	135	195	95	135	80	80	235	126
Fibrinogen (gm/l)	2.32	2.38	3.45	2.26	2.36	3.39	2.27	1.75	1.18	2.32	2.55	1.59	2.40	2.74	1.44	1.18	3.45	2.32
*Fibrinopeptide A (pg/ml)	2.5	0.3	8.2	0.3	1.6	60.0	1.5	1.8	2.1	22.5	2.4	2.4	2.7	0.5	0.3	0.3	60.0	2.1
Resting Euglobulin Clot Lysis Time (Minutes)	100	135	116	92	154	479	47	106	165	38	288	76	140	104	55	38	479	106
Post Venous Occlusion Euglobulin Clot Lysis Time (Minutes)	NA	NA	NA	NA	NA	452	38	70	63	38	244	39	53	62	12	12	452	57.5
Post Insulin Euglobulin Clot Lysis Time (Minutes)	65	86	93	68	98	352	39	55	179	52	108	84	100	152	49	39	352	86

\* Fibrinopeptide A measured at final assessment

TABLE 6 - 4

Established Oral Agent Treated Diabetic Patient Details and Haemostatic Function Results

Subject	K.G.	P.W.	K.F.	A.L.	J.Q.	B.R.	G.M.	A.H.	J.S.	M.D.	M.W.	M.B.	Lowest	Highest	Median
Sex (M/F)	M	M	M	M	M	M	M	M	M	F	F	F			
Age (Years)	38	39	55	56	59	62	62	62	68	45	53	53	38	68	55.5
Weight Index (Kg/m <sup>2</sup> )	18.5	21.53	19.6	20.6	22.5	25.1	24.2	18.8	29.7	24.3	34.8	28.0	18.5	34.8	23.25
Blood Pressure (mm/Hg)	110/60	112/80	130/58	110/70	120/80	120/60	200/100	150/86	160/90	140/80	190/125	130/80	110/60	200/125	135/80
Duration of Diabetes Mellitus (Years)	2	10	3	1	2	10	19	1	3	8	3	0.1	0.1	19	3
Therapy (mg)	G 30 mg	G 30 mg	G 30 mg	G 20 mg	G 15 mg	G 15 mg	C 250 mg	G 20 mg	G 7.5 mg	G 10 mg	Me1000 mg	G 20 mg			
Fasting Insulin (uU/ml)	2.0	7.8	10.2	4.9	NA	4.6	5.0	7.1	4.0	4.0	15.0	14.1	2.0	15.0	5.0
Haemoglobin A <sub>1c</sub> (%)	8.9	11.4	12.0	10.8	7.3	9.1	8.4	11.5	9.5	9.6	6.5	13.0	6.5	13.0	9.5
Fasting Whole Blood Glucose (mmol/l)	10.7	9.0	14.7	15.4	6.0	NA	6.5	11.5	4.2	NA	4.8	17.6	4.2	17.6	9.8
Factor VIII:C (%)	70	100	92	100	118	66	80	84	90	62	100	84	62	118	87
von Willebrand Factor Related Antigen	34	54	56	73	112	NA	72	97	96	70	99	101	34	101	73.0
Ristocetin Co factor Activity (%)	80	74	60	70	150	NA	88	29	84	100	78	NA	29	150	79
Fibrinogen (gm/l)	1.82	3.43	1.81	NA	2.20	2.60	2.63	2.84	2.91	2.70	2.54	NA	1.81	3.40	2.60
Englobulin Clot Lysis Time (Minutes)	112	120	37	NA	361	160	108	180	219	260	580	185	37	580	180

TABLE 6 - 5

Normal Control Subject and Diabetic Patient Median Haemostatic Factor Levels and p Values of Mann Whitney Test Comparing Diabetic Patients with Normal Controls

	Normal Control	All Diabetics	All IDD	NIDD	Est. IDD	New IDD
Number	15	38	24	12	15	9
ECLT (minutes)	101	140.0	130	180	106	185
PV ECLT (minutes)	70	NA	NA	NA	57.5	NA
Fibrinogen (gms/L)	1.69	2.39	2.34	2.60	2.32	2.70
VIII:C (%)	107.5	100.0	110.0	87	110.0	110.0
VWF:Ag (%)	119.0	98.0	113.0	73.0	115.0	111.0
Ristocetin cofactor activity%	89.5	100.0	118.0	79.0	126.0	103.5
FPA (pm/ml)	1.2	NA	NA	NA	2.1	NA
Age (years)	48	52	49	55.5	51	44
Weight Index (%)	23	22.5	22.45	23.25	23.4	19.3

p values of Mann Whitney Test

		All diabetics	All IDD	All NIDD	Est. IDD	New IDD
ECLT (minutes)		0.2730	0.4020	NS	NS	NS
PV ECLT (minutes)		NA	NA	NA	NS	NA
Fibrinogen (gms/L)		0.0005	0.0036	<0.05	<0.05	<0.02
VIII:C (%)		0.8281	0.4977	NS	NS	NS
VWF:Ag (%)		0.8685	0.5449	NS	NS	NS
Ristocetin cofactor activity%		0.2106	0.0311	NS	0.05	NS
FPA pm/ml		NA	NA	NA	NS	NA
Age (years)		0.0874	0.2843	0.05	<0.1	NS
Weight Index (%)		0.7526	0.5627	NS	NS	0.0683

Est = established diabetic patient with microvascular complications

TABLE 6 - 6

Correlations of Age, Weight Index and Blood Pressure with Haemostatic Function  
in Normal Control Subjects (P Values of Spearman's Test of Rank Correlation)

	Age (Years)	Weight Index (%)	Systolic Blood Pressure (mmHg)	Diastolic Blood Pressure (mmHg)
VIII:C (%)	0.3632	0.3541	0.4006	0.5906
vWF:AG (%)	0.3051	0.6806	0.3024	0.3663
Ristocetin Co factor Activity (%)	0.8290	0.5346	0.7497	0.7621
Fibrinogen (gm/l)	0.0106	0.0152	0.0048	0.0210
Fibrinopeptide A (pg/ml)	0.8058	0.4012	0.1398	0.1722
Resting Englobulin Clot Lysis Time (Minutes)	0.6734	0.1029	0.8670	0.7486
Post Venous Occlusion Englobulin Clot Lysis Time (Minutes)	0.0058	0.0425	0.6032	0.5737
% Post Venous Englobulin Clot Lysis Time/Resting Englobulin Clot Lysis Time	0.0193	0.1276	0.5796	0.4617
Age (Years)		0.0960	0.0806	0.0826
Weight Index (%)			0.1212	0.3760
Systolic Blood Pressure (mmHg)				<0.0001

TABLE 6 - 7

Correlations of Age, Weight Index, Blood Pressure and Diabetic Control with  
Haemostatic Function in 37 Diabetic Patients (P Values of Spearman's Test of rank correlation)

	Age (Years)	Weight Index (%)	Systolic Blood Pressure (mmHg)	Diastolic Blood Pressure (mmHg)	Haemoglobin A <sub>1</sub> C (%)	Fasting Whole Blood Glucose (mmol/l)
VIII:C (%)	0.9081	0.2169	0.2146	0.2847	0.0122	0.1979
vWF:AG (%)	0.7536	0.7454	0.8802	0.8497	0.1423	0.1423
Ristocetin Co factor Activity (%)	0.3200	0.3643	0.6252	0.6695	0.2527	0.1814
Fibrinogen (gm/l)	0.7746	0.3601	0.2531	0.1044	0.0347	0.3093
Resting Englobulin Clot Lysis Time (Minutes)	0.2500	0.6055	0.4857	0.0459	0.7652	0.6986
Age (Years)		0.0074	0.0234	0.1083	0.0391	0.3614
Weight Index (%)			0.0005	0.0007	0.0033	0.0559
Systolic Blood Pressure (mmHg)				0.0001	0.0373	0.0025
Diastolic Blood Pressure (mmHg)					0.1330	0.0108
Haemoglobin A <sub>1</sub> C (%)						0.0051

TABLE 6 - 8

Correlations of Age, Weight Index, Blood Pressure and Diabetic Control with  
Haemostatic Function in 24 Insulin Requiring Diabetic Patients  
(Spearman's test of Rank Correlation)

P Values

	Euglobulin Clot Lysis Time	Fibrinogen	Factor VIII Coagulant Activity	von Willebrand Antigen	Ristocetin Co factor Activity
Age	0.0900	0.3216	0.5675	0.6929	0.2256
Weight Index	0.1332	0.5702	0.1982	0.3806	0.6750
Systolic Blood Pressure	0.7413	0.2746	0.3376	0.4740	0.9483
Diastolic Blood Pressure	NS	0.2039	0.3488	0.3590	0.3784
Haemoglobin A <sub>1</sub> C	0.0316	0.0155	0.0591	0.0165	0.6958
Fasting Blood Glucose	0.2972	0.1231	0.1440	0.0879	0.0376

TABLE 6 - 9

The Results of Improved Diabetic Control on Haemostatic Function Tests in 30 Diabetic Patients, Medians and (Ranges) and Results of Wilcoxon Test

	Before	After	p value
HbA <sub>1</sub> C (%)	12.1 8.4 - 24.5	9.8 7.6 - 17.2	<0.0001
FBG (mmol/L)	10.8 4.1 - 77.0	8.1 2.4 - 17.6	<0.05
ECLT (minutes)	130 37 - 479	132 32 - 695	0.1682
Fibrinogen (gm/L)	2.4 1.18 - 3.5	2.36 1.46 - 5.07	0.9399
VIII:C (%)	100 48 - 300	100 54 - 200	0.2187
VWf:Ag (%)	107.5 52 - 320	94.5 46 - 186	0.0065
Ristocetin cofactor activity (%)	103.5 60 - 235	107 62 - 205	0.0268



**Table 6 - 10**

The Results of a Period of Improved Diabetic Control  
on Haemostatic Function 9 Newly diagnosed  
Insulin Requiring Diabetic Patients  
(Medians and Ranges)

Investigation	Visit A Day 0	Visit B Day 14	Visit C Day 40
Fasting Whole Blood Glucose (mmol/l)	11.9 (8.2-77.5)	5.7** (4.0-12.2)	5.6** (4.8-10.3)
Haemoglobin A <sub>1c</sub> (%)	14.5 (11.2-24.5)	12.5* (9.9-17.2)	9.8** (7.4-11.1)
Resting Euglobulin Clot Lysis Time (Minutes)	185.0 (80-330)	522.0* (21-695)	149.0 (60-545)
Fibrinogen (gm/L)	2.73 (1.86-3.5)	2.75 (2.27-5.07)	2.9 (2.13-4.11)
Factor VIII Coagulant Activity (%)	110.0 (76-300)	107.0 (54-200)	79.0 (53-151)
Von Willebrand Related Antigen (%)	111.0 (77-320)	107.0* (54-200)	76.0* (42-100)
Factor VIII Ristocetin Co factor (%)	103.0 (84-190)	113.5 (74-205)	102.0 (82-179)

\* p 0.05

\*\* p 0.01

Wilcoxon Test

**TABLE 6 - 11**

Results of a 90 Day Period of Improved Diabetic Control in 14 Established Insulin Treated Patients; medians (ranges) and p values of Wilcoxon test.

	Before	After	p value
HbA <sub>1c</sub> (%)	10.7 (8.4 - 13.8)	10.1 (7.8 - 13.4)	0.0076
FBG (mmol/L)	10 (4.1 - 22.2)	8.7 (2.4 - 17.6)	0.0640
VIII:C (%)	112 (57 - 230)	102 (74 - 180)	0.5091
vWF:Ag (%)	115 (52 - 204)	96 (55 - 186)	0.1026
Ristocetin cofactor activity (%)	135 (80 - 255)	98 (80 - 170)	0.1422
Fibrinogen (gm/L)	2.36 (1.18 - 3.45)	2.16 (1.46 - 2.63)	0.3451
ECLT (minutes)	111 (38 - 479)	85 (32 - 400)	0.0393
PV ECLT (minutes)	93 (49 - 352)	72 (28 - 145)	0.2487

TABLE 6 - 12

Serial Fibrinopeptide - A (FPA) levels in  
15 established Diabetic Patients  
(pg/ml)

Visit 1 F.P.A.	Visit 2 F.P.A.	Visit 3 F.P.A.	Visit 4 F.P.A.	Retinopathy S/D
5.8	N.A.	4.9	2.5	S
21.3	8.5	3.9	8.2	D
4.6	3.4	1.9	0.3	D
5.3	1.5	6.8	1.5	S
2.1	1.8	1.5	1.8	S
4.5	1.6	1.4	2.1	S
6.5	1.6	2.0	2.4	S
1.2	1.4	1.1	0.3	S
6.0	4.5	60.0	2.4	S
4.4	3.5	1.1	0.3	S
NA	12.2	7.3	1.6	S
16.5	60.0	60.0	60.0	D
2.5	2.1	3.7	22.5	S
1.2	1.2	1.3	2.7	S
1.0	0.3	0.8	0.5	S
Abnormal Results 9.0	5.0	7.0	3.0	

N.A. = Not Assessed

TABLE 6 - 13

Medians and Ranges of Haemostatic Function Factors on Treatment with BHI and PPI and Median Changes occurring on Treatment with each Insulin,  $\Delta$ BHI and  $\Delta$ PPI.

	BHI	PPI	p value	$\Delta$ BHI	$\Delta$ PPI	p value
ECLT (minutes)	108 (33-387)	111 (42-495)	NS	-9.5	-1.0	NS
PV ECLT (minutes)	39 (8-517)	45 (18-350)	NS	0	-4.5	NS
PI ECLT (minutes)	65 (36-295)	80 (45-480)	NS	-14.0	+11.0	NS
Fibrinogen (gm/L)	2.16 (1.54-3.49)	2.1 (1.17-3.66)	NS	+0.02	-0.03	NS
VIII:C (%)	110 (64-180)	86 (70-220)	NS	+4.0	-11.0	NS
VWF:Ag (%)	95 (45-171)	103 (38-158)	NS	-2.0	-9.0	NS
Ristocetin cofactor (%)	97 (72-205)	115 (86-188)	NS	0.0	+7.0	NS
FPA (pmol/ml)	2.1 (0.3-60)	2.0 (0.3-60)	NS	+0.15	-0.2	NS

TABLE 6 - 14

The Effects of Treatment with Insulin on Haemostatic Function in NIDD: a) Results at Entry to Study, b) Results After a 90 day Run-in Period of Improved Control on Oral Agents, c) Results after 90 days' Treatment with Insulin.

		JS	GM	MD	Mean
Hb <sub>1c</sub> (%)	a	9.5	8.4	9.6	9.2
	b	7.8	7.9	8.6	8.0
	c	7.3	9.8	9.7	8.9
FBG (mmol/L)	a	4.2	6.5	NA	
	b	6.5	7.4	5.7	6.5
	c	7.9	8.7	7.7	8.1
VIII:C (%)	a	90	80	62	77.3
	b	80	170	64	104.6
	c	62	115	80	85.7
vWf: Ag (%)	a	96	72	74	80.7
	b	95	65	46	68.7
	c	85	101	59	81.7
Ristocetin cofactor activity (%)	a	84	88	100	90.6
	b	84	90	100	91.3
	c	90	100	105	98.3
Fibrinogen (gms/L)	a	2.56	2.63	2.70	2.63
	b	2.95	2.36	2.33	2.54
	c	2.56	2.88	2.29	2.57
ECLT (minutes)	a	97	108	260	155
	b	397	167	232	259
	c	195	161	238	198

## CHAPTER 7

### PERIPHERAL BLOOD FLOW RESULTS

#### 7-1 INTRODUCTION

Blood flow is determined by extrinsic (neural and hormonal) control systems, intrinsic autoregulatory capacity and the rheological properties of blood, all of which may be altered by diabetes mellitus. It has been postulated that high blood flow rates and loss of capillary flow autoregulation might result in "overperfusion" and this may cause capillary damage and lead to the development of microangiopathy (Parving et al, 1983 [b]). Current diabetic practice is based on the premise that the development of microangiopathy is in some way related to the duration and degree of hyperglycaemia, but there is little evidence that, once diabetic complications have occurred, good control of blood glucose levels will prevent further damage. Indeed, in some instances, paradoxical deterioration of microvascular diabetic complications has been noted on the institution of strict regimes which lower blood glucose levels (Hooymans et al, 1982). Alterations in blood flow with changes in glycaemia might contribute to these undesirable effects, and it would appear that a more thorough understanding of the changes in blood flow regulation, and the relationship of these changes to quality of control and treatment of diabetes mellitus is required before a rational treatment policy can be formed.

#### 7-2 AIMS OF THE STUDIES OF PERIPHERAL BLOOD FLOW

These were to establish whether:

- i) there was any specific defect of peripheral blood flow associated with the presence of diabetes mellitus
- ii) there was any influence of age, duration of diabetes mellitus and diabetic control on peripheral blood flow
- iii) there was any effect of improved blood glucose control on peripheral blood flow
- iv) treatment with biosynthetic human insulin improved the blood flow in the lower limb in diabetes mellitus.

In view of the marked heterogeneity of diabetic patients, blood flow in Type I insulin dependent patients was studied, and compared with blood flow in Type II non-insulin dependent diabetic patients. Blood flow in newly diagnosed diabetic patients without complications was also compared with blood flow in established diabetic patients with

known microvascular disease. A control group was also studied. The blood flow parameters studied were:

- i) Resting calf flow (RCF)
  - ii) Resting foot flow (RFF)
  - iii) Reactive hyperaemia, following the ischaemic stress induced by a 4 minute arterial occlusion with;
    - a) time taken to attain peak flow values following release of the arterial tourniquet (Time Peak Flow; TPF)
    - b) peak flow value attained during the reactive hyperaemia phase (Peak Post Stimulus Flow; PPSF) and also PPSF-RFF
    - c) time for the peak flow to fall to half its original value following release of the arterial tourniquet ( $T_{50}$ )
    - d) arterial debt: this represents the deficit in blood flow induced by artifactual arterial occlusion and is calculated by multiplying resting foot flow (RFF) per minute by the time in minutes of arterial occlusion (Art Debt).
    - e) the percentage of the arterial debt paid off during the first 30 seconds of blood flow after release of the arterial tourniquet, (0-30%): this is calculated by dividing the blood flow in the first 30 seconds of the reactive hyperaemia response by the arterial debt and multiplying by 100
- $$0-30\% = (\text{Flow in 0-30 seconds} / \text{art debt}) \times 100$$

### 7-3 METHODS AND STATISTICAL ANALYSIS

Blood flow measurement methods are described in chapter 3. The results were analyzed using non-parametric statistical methods because of the small numbers and the non-symmetrical distribution of the results. Different groups were compared using the Mann Whitney U test, and the effects of treatment with purified porcine and biosynthetic human insulins were examined using the Wilcoxon rank signed test, and the correlations of flow measurements with age, duration of diabetes, diabetic control and insulin levels were examined using Spearman's test of rank correlation.

### 7-4 DETAILS OF THE SUBJECTS STUDIED

#### 7-4-1 Normal Subjects

Details and results of the 16 normal subjects (6 female, 10 male) are shown in Table 7-1. Many of these subjects also participated in the studies of haemostatic factors. The median age was 47.5 (range 24 - 62 years). There were no smokers in this group and all the subjects were in good health and gave no history of vascular disease apart from R.A. who had a history of previous myocardial infarction, but was asymptomatic at the

time of study. Examination of the peripheral pulses was normal. These subjects were generally studied during the afternoon and were examined once.

#### 7-4-2 Newly Diagnosed and Established Insulin Treated Diabetic Patients

The diabetic patients studied included 9 newly diagnosed patients with Type 1 diabetes mellitus of whom 6 were re-examined after a period of improved diabetic control. Full medical details of these patients, who had no evidence of diabetic vascular disease are given in Chapter 6, Table 6-2. Peripheral blood flow results on these patients are shown in Table 7-2. In addition, 28 established diabetic patients with microangiopathy were studied. This included 15, Type I, insulin dependent diabetic patients who took part in the double blind crossover study of the effects of treatment with purified porcine and biosynthetic human insulins, details of whom are given in Chapter 6, Table 6-3. Peripheral blood flow results are given in Table 7-2.

#### 7-4-3 Established Diabetic Patients Treated with Oral Hypoglycaemic Agents

The remaining 13 established patients were Type II diabetics, treated with oral hypoglycaemic agents. Medical details of 12 of these patients who also took part in the haemostatic factor studies are shown in Chapter 6, Table 6-4. Peripheral blood flow results are given in Table 7-3. The additional Type II patient (D.J.), was a 54 year old male with a ten year known duration of diabetes, treated with glibenclamide and metformin, who had proliferative retinopathy.

In summary, a total of 37 diabetic patients were studied, median age 52, range 18-68 years. Ten patients had no evidence of microvascular disease, 17 had mild retinopathy and 10 had required laser photo-coagulation treatment for proliferative disease. Blood flow measurements in the diabetic patients were performed in the morning, post prandially between 9.30 and 10.45 am. Full details of blood flow measurements are given in Chapter 3. Six of the newly diagnosed patients were examined twice, at diagnosis and after a period of improved control. The 15 patients who participated in the double blind BHI and PPI insulin crossover study were examined four times;

- 1) at entry to the study
- 2) after a three month run-in period
- 3)&4) after treatment with each trial insulin respectively for three months.

The 13 established Type II patients were examined once.



## 7-5 RESULTS OF BLOOD FLOW MEASUREMENTS IN NORMAL VOLUNTEER SUBJECTS.

Results are shown in Table 7-1 and summarized in Table 7-4. Resting blood flow rates in both the calf (RCF), range 1.6 - 7.6 mls per 100 mls of tissue, and foot (RFF), range 0.09 - 4.2 mls per 100 mls of tissue, showed wide individual variations and in 3 subjects RFF was barely recordable and arbitrarily given a value of 0.09 mls per 100 mls of tissue as the lowest recordable value of flow was 0.1ml/100 mls of tissue. The median value of RCF was 3.8 mls per 100 mls of tissue and the median value of RFF 0.7 mls per 100 mls of tissue. The plethysmograph measures changes of volume occurring with arterial inflow and the large non-distensible bony component of the forefoot probably accounts for the lower recorded RFF, as compared with the more distensible calf (see discussion, 7-8-2). Very low recorded RFF made calculation of arterial debt following arterial occlusion impossible, and also affected the accuracy of the calculation of peak post stimulus flow rates - resting flow rates (PPSF-RFF) and percentage of arterial debt paid back in the first 30 seconds of reactive hyperaemia (0-30%). When RFF was very low i.e. 0.09 mls per 100 mls of tissue, 0-30%, was often apparently several hundred percent but was given an arbitrary value of 500%. Despite attempts to achieve acclimatization by the resting of subjects for 30 minutes in a constant temperature room before commencing measurements, foot temperature varied greatly between individuals and, as expected, warmer feet exhibited higher resting flow values, ( $p=0.0007$ ), see table 7-5.

After arterial occlusion, Peak Post Stimulus Flow rates (PPSF) were rapidly achieved, and Time to Peak Flow (TPF) was always within 9 seconds, indicating that the low flow rates in some of the normal control subjects were not due to impaired perfusion secondary to undiagnosed atherosclerosis. The range of 0-30% was 29 - 500%, with a median value of 67.2%. The 0-30% value of 29% may be an inaccurate estimate as it was obtained on a 28 year old female with barely recordable foot flow. TPF was normal in this subject. Mean and median values of 0-30%, omitting patients with barely recordable RFF (and hence estimated 0-30% values) were also calculated. The mean  $\pm$  SD, (0-30%) was 87%,  $\pm$  61.6%,  $n=13$ , and the median value 51%. Vascular reactivity following ischaemic stimulus, was also highly variable between individuals, as witnessed by the wide range of half-life of reactive hyperaemia following arterial occlusion ( $T_{50}$ ) from 7 to 62 seconds, median 24 seconds.

Whilst metabolic, emotional and environmental factors might all contribute to these wide variations in blood flow, so might structural changes in the vessels such as loss of elasticity as associated with increasing age. On analysis of the correlation of age with the various blood flow measurements the only significant finding was unexpected (see Table 7-5). Increasing age was associated with a greater 0-30%, ( $p=0.0044$ ), the

reverse of what might be expected with a loss of vessel elasticity due to age. However numbers are small, only 2 patients being over 50 years of age, and the inaccuracy in recording very low resting foot flow may mean that the association of increased 0-30% with increasing age may be a spurious finding. A typical blood flow recording is shown in Figure 3.4.

## 7-6 RESULTS OF PERIPHERAL BLOOD FLOW MEASUREMENTS IN DIABETIC PATIENTS

### 7-6-1 All diabetic patients

Details on individual patients are given in Tables 7-2 and 7-3. Results are summarized in Table 7-4. There were large individual variations in blood flow, RCF ranged from 1.6 - 5.3 mls per 100 mls of tissue and RFF from 0.09 - 2.7 mls per 100 mls of tissue. RFF was barely recordable in 4 patients and given an arbitrary value of 0.09. Diabetes mellitus is commonly associated with high resting blood flow rates, particularly when neuropathy is present (Ward, 1982). However, in this group of 37 diabetic patients, the minimum resting flow rates were comparable and maximum resting flow rates in the foot and calf were in fact less than those of the normal subjects. The median RCF, 2.5 mls per 100 mls of tissue and RFF, 0.8 mls per 100 mls of tissue were not significantly different from normal volunteer resting blood flow (RCF 3.8 and RFF 0.7 mls per 100 mls of tissue respectively). As with the normal subjects, higher RFF was associated with warmer feet,  $p=0.0214$ , (see Table 7-6). It was noted that the established diabetic patients had cooler feet (median  $28.9^{\circ}\text{C}$  versus  $31.8^{\circ}\text{C}$ ) than normal subjects, although this was not statistically significant. Toe-pad skin temperature was not recorded in the newly diagnosed patients. Arterial insufficiency is a cause of cool feet, but there was no obvious correlation of temperature and other indices of arterial insufficiency such as delay in TPF or low values of 0-30%. Whether slight differences in environmental temperature or of adaptation of foot temperature to the temperature in the constant temperature room accounts for differences in skin temperature between diabetic subjects and normal volunteers is not known. Temperature is an important determinant of resting skin blood flow.

A striking finding was, that compared with the normal subjects, the corrected median peak blood flow value following arterial occlusion of the foot (PPSF-RFF) was 35% lower in diabetic patients, (median diabetic 6.2 versus normal 9.5 mls per 100 mls. of tissue ( $p < 0.0001$ )). The median TPF of 4.8 seconds, was identical in diabetic and normal subjects, but TPF was more than 9 seconds in 8 diabetic patients whereas it was always less than 9 seconds in the normal volunteers. Reduced peak flow following arterial occlusion and delay in TPF could both be interpreted as indicating obstructive arterial disease, but it was noted that delayed TPF was found in diabetic

patients with resting flow rates amongst the highest of those measured. Delay in TPF was also noted in 3 out of 9 newly diagnosed diabetic patients. There were no significant differences of  $T_{50}$ , median value 21.6 seconds (range 4.6 - 122 seconds) nor any evidence of reduced 0-30%, median value 72.6% (range 22.5 - 500%) in the diabetic patients. Prolongation of  $T_{50}$  and reduced 0-30% are also features of arterial insufficiency due to atherosclerosis. Atherosclerosis of the larger vessels is thus unlikely to be the only explanation of reduced PPSF-RFF and delay in TPF in the diabetic patients. Overall the results suggest that the distensibility of diabetic vessels might be reduced either because of an intrinsic vessel defect or because of alterations in the mechanisms controlling vasodilation, whether neural, hormonal or metabolic. Furthermore, it would appear that this abnormality occurs early in the history of diabetes mellitus. Another possible explanation of delay in TPF and reduced PPSF-RFF is that increased blood viscosity in diabetes results in impaired perfusion of vessels (see chapter 5).

#### 7-6-2 The Influence of Age, Duration of Diabetes Mellitus and of Diabetic Control on Peripheral Blood Flow

It has been suggested that flow rates decline with increasing duration of diabetes mellitus due to loss of vessel elasticity and the development of atherosclerosis (Christensen, 1969). However, this author did not find any statistical correlation of age nor of duration of diabetes with RCF, RFF, TPF, PPSF-RFF,  $T_{50}$  or 0-30% values to support the notion of a progressive vascular lesion (see Table 7-6). Duration of diabetes is however difficult to assess, particularly in Type II diabetic patients who may have a long history of symptoms of diabetes mellitus before presentation and diagnosis. There was no significant difference in peripheral blood flow values between newly diagnosed and established diabetic patients (see Table 7-4) although it was noted that Type II established diabetic patients had the lowest median resting flow values and PPSF-RFF. Eight out of 13 (61%) of Type II patients were over the age of 50, but only 10 out of 24 (42%) Type I insulin dependent diabetic patients were over 50 years. (Only 2 out of the 16 (12%) of normal control subjects were over 50 years). The trend towards lower PPSF-RFF in the Type II patients may reflect increasing age and loss of vessel elasticity. There was a weak correlation of lower PPSF-RFF with increasing age in the Type II diabetic patients,  $p = 0.0609$ , but there was no evidence of reduced repayment of arterial debt with increasing age. Diabetic control, as assessed by  $HbA_{1C}$  and blood glucose levels, taken at the time of blood flow recordings, was not associated with any significant trend in blood flow measurements although reversible high flow rates in the forearm are reported in association with poor diabetic control (Gundersen, 1974), see discussion. The interaction of multiple other determinants of blood flow may have obscured any relationship, see Table 7-6.

### 7-6-3 Insulin and Peripheral Blood Flow

As insulin may be a vasodilator (Tooke et al, 1980), the results were examined to see if insulin levels at the time of blood flow measurement had any influence on blood flow. Insulin levels were measured in the 15 established Type I diabetic patients and in 4 other patients treated with oral agents, but there was no association with blood flow measurements (see Table 7-10).

### 7-6-4 Peripheral Nerve Function and Peripheral Blood Flow

Diabetic neuropathy is associated with marked disturbances of blood flow and the results were examined to establish whether there was any association of peripheral nerve function, as measured by Common Peroneal Nerve sensory and motor conduction times, and peripheral blood flow measurements. Peripheral nerve conduction studies were performed in 18 established diabetic patients within a week of the peripheral blood flow measurements. Detailed studies of autonomic nerve function were not performed because of lack of time and equipment. There was no association of higher resting blood flow rates and impaired peripheral nerve function as assessed by nerve conduction times. However, the Common Peroneal nerve f latency value showed an association with the TPF following arterial occlusion in the foot ( $p = 0.0484$ ), indicating that neural factors may influence peripheral blood flow in diabetes mellitus (see Table 7-10). Results of peripheral nerve function are presented fully in chapter 8, Table 8-1.

### 7-6-5 The Effect of Improving Blood Glucose Control on Peripheral Blood Flow in Newly Diagnosed Patients

Nine patients were examined and median flow values were similar to those of established diabetic patients with the same wide range of blood flow values (see table 7-4). The effects of improvement of diabetic control on peripheral blood flow was examined in 7 patients. Three of them were examined both before treatment at diagnosis and 30 days later and the others were examined both after starting insulin treatment and 30 days later. Results are shown in Table 7-7. Improvement of diabetic control was confirmed by a fall in  $HbA_{1C}$  levels in each case ( $p < 0.05$ ). In those patients who were examined before diagnosis and after treatment, the consistent changes noted were an increase in the PPSF - RFF following arterial occlusion in the foot and a shortening of TPF. These changes could be interpreted as representing an increase in vessel distensibility, or perhaps representing changes in the neural control of vasodilation. It is known that nerve conduction is abnormal in newly diagnosed diabetic patients and returns to normal on treatment with insulin (Ward et al, 1971). A relationship between TPF and nerve conduction has been demonstrated by the author in established diabetic patients (see above, 7-6 a) providing support that neural control

mechanisms may affect vasodilation. Alternatively, alterations in blood viscosity may have affected flow. In the time period (40 days) over which the new diabetic patients were examined, rehydration will have resulted in a fall of haematocrit but it is unlikely that any change in fibrinogen, the other main determinant of whole blood viscosity, will have occurred. The effect of rehydration on TPF and PPSF have not been investigated elsewhere. There were no consistent changes in duration of reactive hyperaemia or of resting flow values. In the patients examined after starting treatment for diabetes, there were no significant changes during the period of improved control. This indicates that changes in the regulation of peripheral blood flow in acute diabetes mellitus, of whatever cause, are rapidly reversible with treatment.

#### 7-6-6 The Effects of Treatment with Biosynthetic Human Insulin and Purified Porcine Insulins on Peripheral Blood Flow in 15 established Type 1 Diabetic Patients

Fifteen diabetic patients with microvascular disease took part in a double-blind trial of treatment with BHI and PPI. Details of the protocol are given in chapter 2-4. Peripheral blood flow in these 15 patients was measured on 4 occasions, at entry to the study and after a 3 month period of optimized diabetic control on conventional multiple subcutaneous insulin regimes and after 3 months treatment with each of the trial (BHI and PPI) insulins. Results are shown in Table 7-8. Blood flow in the foot and calf were similar to those of newly diagnosed Type I and established Type II diabetic patients. A small improvement in diabetic control was achieved during the run-in period, median fall in HbA<sub>1</sub>C% 1.0% ( $p < 0.05$ ). This improvement of diabetic control was associated with an apparent small increase of RFF ( $p < 0.04$ ), but foot temperature at the time of the second recording was also noted to be significantly higher, 30 °C versus 28.1 °C ( $p = 0.0119$ ). Thus environmental factors may have influenced flow. Alternatively, higher flow rates might have resulted in warmer feet. On treatment with BHI and PPI there was no change in the overall control of diabetes mellitus as assessed by HbA<sub>1</sub>C and fasting blood glucose levels. Similarly there were no changes in any of the parameters of peripheral blood flow assessed. Treatment with BHI had no detectable effects on peripheral blood flow in the calf and foot. Results are summarized in Table 7-8.

#### 7-7 RESULTS OF LASER DOPPLER FLOW STUDIES

Towards the end of the studies the opportunity to measure skin blood flow with a Laser Doppler Flow Meter became available. Toe pad flow in response to arterial occlusion of the foot was examined in 9 of the diabetic subjects (all established insulin-requiring) and 4 of the normal volunteers. Full details of methods used are given in Chapter 3-5. Measurements were made TPF following arterial occlusion and

results are shown in Table 7-9. A simultaneous recording of plethysmographic TPF was made. Although numbers are small, there was no overlap in the the TPF's measured by the laser doppler meter of diabetic individuals and those of normal volunteers, all the former being longer. Plethysmographic TPF's were similar but in normal subjects and diabetic patients TPF's measured by the Laser Doppler Flow Meter are notably longer than TPF values obtained using plethysmographic measurements. In normals the median laser doppler TPF was 19.8 seconds, 4 times longer than the plethysmographic TPF whilst in diabetic patients the median laser doppler TPF was 55.2 seconds, some 13 times longer than the plethysmographic TPF. These findings suggest that there is a defect in the vasodilation of small blood vessels (arterioles and capillaries) in diabetes mellitus in response to an ischaemic stimulus. Abnormalities of blood flow in small vessels appear to be greater than flow abnormalities in large vessels as measured by plethysmography. These changes could be explained by decreased small vessel distensibility, either functional or mechanical, or impaired vessel perfusion due to alterations in blood viscosity.

## 7-8 DISCUSSION

### 7-8-1 Factors Influencing Blood Flow Measurements.

Although the main manifestations of diabetic complications are vascular, peripheral blood flow has been less extensively studied than many other aspects of diabetes mellitus. Special equipment is needed and measurements of peripheral blood flow are time-consuming. In addition, there are many practical problems related to the extreme lability of blood flow, and the critical influence of such factors as the emotional state of the patient, and ambient and body temperatures. Temperature is particularly important with regard to the flow in the medium sized vessels measured by the plethysmograph (Felder et al, 1954). Achieving identical conditions for follow-up and comparison studies presents many difficulties. In the studies presented here, the diabetic patients were studied over a 9 month period, during which ambient temperatures varied greatly. Patients were in the hospital for approximately one and a half hours, of which half an hour was spent in a constant temperature room, before blood flow was measured. They were always studied at the same time, and at the same interval after an identical breakfast and usual morning medication. Despite these attempts at standardization, it was noted that the median foot temperature in diabetics, who were predominantly recruited during the winter months and early spring, was cooler (though not significantly) than that of the normal controls, who were generally studied in autumn. The median diabetic foot temperature was significantly higher at the second visit, in those patients participating in the double-blind crossover insulin treatment trial. The normal control subjects were studied in the afternoon, on the assumption that diurnal variations in flow were unimportant, but the cooler

temperature of the diabetic feet may also reflect this time difference. Slower adaptation of skin temperature to changes in environmental temperature is a feature of diabetes (as reviewed in section 1-7-2), and although foot temperatures were not significantly different, an even longer period of acclimatization in the constant temperature room may have been desirable.

#### 7-8-2 Comparison of Blood Flow in Diabetic and Normal Volunteers

Blood flow in the feet was studied because this is the extremity in which diabetic vascular problems occur. Resting flow rates in the all diabetic patients were not significantly different from normal. Other plethysmographic studies of blood flow in diabetes have generally examined the calf and forearm, predominantly measures of muscle blood flow. In poorly-controlled, short-duration diabetes, increased resting forearm blood flow rates have been noted (Christensen, 1970; Gundersen, 1974). Less information on blood flow in the foot is available. In an early study by Mendolowitz and colleagues (1953) decreased diabetic toe flow was found, and was thought to represent asymptomatic vascular disease. However in the author's study, of poorly controlled newly diagnosed diabetic subjects who will be free of chronic diabetic damage, resting flow values were also generally low and in some cases barely recordable. Whilst these results are apparently at odds with the studies of forearm flow by Christensen (1970) and Gundersen (1974), plethysmographic recordings of foot blood flow may not be equivalent to measures of forearm muscle flow. The foot has a larger non-distensible bony component, and the proportion of skin to muscle is greater. Foot flow measurements may be less sensitive than both those of the calf and the forearm, and the low flow rates may be a spurious finding, reflecting small numbers. Another possibility is that the abnormalities of blood flow were largely reversed by the time the patients were examined, as only 3 patients were examined before commencing treatment for diabetes mellitus. Gundersen (1974) on the other hand, examined forearm blood flow in diabetic subjects whose usual diabetic treatment had been deliberately stopped some days before.

In the established diabetic patients examined by the author, there was no association between resting blood flow values and diabetic control as measured by HbA<sub>1c</sub> levels or blood glucose. Long duration of diabetes mellitus has been previously associated with lower resting flow values (as reviewed by Christensen 1972). In the author's studies, the opposing effects of hyperglycaemia and duration of diabetes mellitus may have effectively cancelled each other out. A small improvement of diabetic control, did not result in any significant change in resting blood flow values. This apparent discrepancy from the results of other studies, (Gundersen, 1974), may reflect on the small improvement of diabetic control achieved. However, the aim of the author's

study was to establish the effects of improved diabetic control, as can be achieved in clinical out-patient practice.

The main difference, between normal control and all diabetic subjects, was a marked reduction of peak post stimulus flow (PPSF) following arterial occlusion in the feet. Reduced PPSF in diabetic patients was also noted in a study by Cunningham et al, (1983). Both cooler foot temperature and reduced PPSF could be manifestations of vascular disease, but reduced PPSF was also noted in newly diagnosed Type I diabetic patients studied by the author. The reduction of PPSF in newly diagnosed diabetic patients and subsequent improvement after starting treatment, suggests the presence of a reversible abnormality of vasodilation. In addition, poor vasodilation responses in diabetes have been reported on CO<sub>2</sub> challenge with regard to cerebral blood flow (Dandona et al, 1978), in toe flow after ganglion blockage (Megibow, 1949 as reviewed by Christensen, 1972) and with local heating or needle injection trauma (as reviewed by Tooke, 1986). In young patients without neuropathy, it has been noted that haemoconcentration in the dependant foot on quiet standing does not occur to the same extent as in normal subjects (Rayman et al, 1986). These abnormalities have been suggested as evidence of an intrinsic defect of pre-capillary vasoconstriction in diabetes.

Other plethysmographic measurements of reactive hyperaemia, the time taken to reach peak post stimulus flow values (TPF) and the duration of reactive hyperaemia as measured by its half life (T<sub>50</sub>) were not abnormal in diabetes mellitus, although a shortening of TPF was noted after starting treatment with insulin in 2 of the 3 newly diagnosed patients, the other remaining unchanged. The TPF in the toe pad following arterial occlusion of the foot was also measured in 9 established diabetic patients and in 4 normal controls using a Laser Doppler Flow Meter which became available towards the end of the studies. The Laser Doppler Flowmeter measures microcirculatory as opposed to arterial flow. The thickness of the overlying skin keratin determines the site and hence type of skin blood vessel in which flow is measured. In the diabetic patients, TPF measured by the Laser Doppler Flowmeter was considerably prolonged and this delay was greater than plethysmographic TPF values suggesting that the principal abnormality of flow regulation is to be found in the small vessels. Thus there is evidence of increased vascular resistance or of decreased distensibility of the vascular bed, particularly of the small vessels, in diabetes.

### 7-8-3 Autoregulation of Blood Flow in Diabetes mellitus

Anecdotal reports of deterioration in diabetic retinopathy, following dramatic improvement in diabetic control on commencing treatment with continuous subcutaneous insulin infusion, have raised doubts about the efficacy of quickly



lowering blood glucose near normal. The theory is that sudden reversal of the high flow rates associated with poor diabetic control may be deleterious to ischaemic retina, dependent on high perfusion rates for adequate nutrition.

Whilst the studies presented in this thesis provide no evidence of high perfusion rates in diabetes mellitus, nor of dramatic changes in resting flow rates on modest improvement of diabetic control, much evidence of overperfusion is available. Parving et al (1983 [b]) proposed that the late structural microvascular abnormalities in diabetes mellitus are a consequence of early haemodynamic changes that lead to increased capillary flow and pressure. It was suggested that high glucose levels might in some way interfere with the normal autoregulation of flow. Failure of normal autoregulation of capillary blood flow in the face of changing perfusion pressures may then lead to high capillary pressure and the development of basement membrane thickening and vessel degeneration. In support of this theory, they cited a mass of evidence including; reports of the reversible increases in glomerular filtration rates and urinary excretion of proteins in newly diagnosed diabetic patients, which they postulated were due to increased renal plasma flow and increased glomerular pressure; the protection from retinopathy which appears to be afforded by carotid stenosis and hence lower perfusion pressure of the retinal vessels (Dandona et al, 1978); and similarly the protection from nephropathy by renal artery stenosis (Behrendt & Duane, 1970). Indeed, the increased severity of diabetic vascular lesions in the foot (where hydrostatic pressure is greater) compared with the arm, would also support this theory of loss of normal flow autoregulation in the face of changing perfusion pressures in diabetic patients.

Loss of normal autoregulation, may also result in inappropriately low blood flow. Studies indicate that nailfold capillary flow velocity in diabetic patients is lower than normal (Tooke & Tymms, 1983). It has been suggested that in diabetic patients flow through arteriovenous anastomotic shunt pathways is increased at the expense of skin blood flow. Studies of the oxygen content of venous blood (Boulton et al, 1982) and of Doppler sonographic studies (Edmonds et al, 1981) of diabetic feet provide further evidence for the presence of arteriovenous shunting in the diabetic neuropathic foot. Whether reversible shunt flow occurs in patients without neuropathy, and is a feature of transient autonomic nerve dysfunction associated with poor control, or is a physiological response to the raised metabolic rate and need for heat dissipation in uncontrolled diabetes, is unknown.

#### 7-8-4 Neural Factors and Blood Flow

Direct measurements of the peripheral autonomic nerve conduction times are not possible. The author did not have the facilities and time for detailed testing of autonomic nerve function. Measurements of the Peroneal Nerve conduction times were

used as a guide to the state of the peripheral nerves in the diabetic patients (although it cannot be assumed that these measurements accurately reflect autonomic nerve function). No associations of Peroneal Nerve conduction times and resting blood flow values were found in this author's studies but only two patients had symptomatic diabetic neuropathy. However, there was an association of the TPF with Peroneal 'f' latency times, suggesting that impaired vasodilation may partly related to alterations of vessel neural control mechanisms.

#### 7-8-5 The Influence of Insulin, Diabetic Control, Blood Viscosity and Treatment with Biosynthetic Human Insulin on Peripheral Blood Flow.

Other factors thought to influence blood flow in diabetes include i) raised glucose levels, which in animal experiments have been associated with retinal vessel vasodilation and increased blood flow (Atherton et al, 1980), ii) insulin, which appears to act as a micro-vasodilator in the skin (Tooke et al, 1985) and iii) duration of diabetes mellitus (as reviewed by Christiansen 1972), but in these studies no associations of glucose or insulin levels or duration of diabetes mellitus with blood flow measurements were found. Blood rheological properties have also attracted attention as influencing flow. The relationship of whole blood viscosity with peripheral blood flow was examined in Chapter 5. Whilst peak flow values were statistically related to whole blood viscosity in normal control subjects, no such relationship was found in diabetes mellitus. It seems unlikely that the minor changes in blood viscosity described in these studies have influenced blood flow.

Similarly insulin levels were not specifically associated with any abnormality of blood flow and it is hardly surprising that the crossover study of the effects of treatment with biosynthetic human and purified porcine insulins on peripheral blood flow provided no differences. Certainly treatment with biosynthetic human insulin does not confer any advantage as regards peripheral blood flow.

#### 7-8-6 Conclusions

The belief that abnormalities of blood flow regulation in the microcirculation might contribute to vessel damage is gaining increasing support although the determinants of these abnormalities remain speculative. In the studies presented here, evidence of impaired vasodilatory responses after ischaemic stress, occurring early in the history of diabetes supports the concept of a functional microangiopathy preceding small vessel degeneration in diabetic patients. The opposing effects of the many variables influencing blood flow make interpretation difficult and examination of the effects of specific factors e.g. glucose or insulin levels, would be better achieved by glucose clamp type experiments in patients matched for age, sex and duration of disease. Further studies are required to determine the site of these abnormal flow patterns i.e.

whether the lesion is an intrinsic vessel malfunction, or due to abnormal neural control mechanisms or indeed a physiological response to the altered metabolic milieu of diabetes mellitus. The combined use of Plethysmographic and Laser Doppler measurements of blood flow may help distinguish between large and small vessel damage. However, as the principal defect in diabetes is thought to reside in the small vessels, studies of skin blood flow using the Laser Doppler Flowmeter may prove more rewarding. Further research into the exact nature of the vessels measured by the Laser Doppler Flowmeter at different skin sites are required because of the influence of the thickness of the overlying layer of keratin.

**APPENDIX****TABLES TO CHAPTER 7**

TABLE 7 - 1

Normal Control Subjects  
Peripheral Blood Flow Results

	H.Y.	R.C.	S.J.	M.L.	G.O'L	M.T.	R.A.	P.C.	R.H.	R.D.	P.O'L	C.W.	R.B.	E.M.	J.Pe.	D.Pr.
Sex	F	F	F	F	F	F	M	M	M	M	M	M	M	M	M	M
Age (Years)	28	49	48	48	44	58	61	47	62	50	48	43	24	26	31	28
Resting Calf Flow	2.5	2.0	3.2	4.4	7.6	5.0	4.5	3.6	1.9	4.6	5.0	4.0	2.2	4.7	3.5	1.7
Resting Foot Flow	0.09	0.7	2.3	0.5	4.2	0.5	0.8	2.7	0.09	0.09	2.5	2.6	0.8	3.0	0.5	0.5
Time to Peak Flow (Seconds)	6.0	6.0	7.2	3.6	0.0	8.4	0.0	8.4	6.0	7.0	4.8	9.0	3.6	0.0	4.8	4.2
Peak Post Stimulus Flow	3.0	14.5	9.5	7.0	18.4	9.9	24.5	13.5	12.5	7.5	13.2	13.5	9.0	12.5	8.5	8.5
Peak Post Stimulus Flow ( Resting Flow)	3.0	13.8	7.2	6.5	14.2	9.4	23.7	10.7	12.5	7.5	10.7	10.9	8.2	9.5	8.0	8.0
T <sub>50</sub> (Seconds)	12.0	24.0	26.4	13.2	50.4	43.2	16.8	40.8	24.0	7.5	62.4	55.2	14.0	14.0	11.0	7.0
0-30%	29	155	38	82	42	190	216	39	500	500	52.5	48	83	35	100	50
Temperature (°C)	25.5	27.8	32.8	30.6	32.4	28.8	32.0	30.0	30.0	30.9	34.2	32.8	33.1	32.4	31.6	31.3



TABLE 7 - 3

Peripheral Blood Flow Results of 13 Oral Agent Treated  
Diabetic Patients

	M.D.	M.W.	J.F.	F.W.	K.G.	P.W.	D.J.	K.F.	J.Q.	A.H.	G.M.	K.L.	J.S.
Sex	F	F	F	F	M	M	M	M	M	M	M	M	M
Known Duration of Diabetes Mellitus (Years)	8	3	3	15	1	2	10	3	2	1	19	5	3
Haemoglobin A <sub>1c</sub> (%)	9.6	6.5	8.9	11.4	8.9	12.2	10.4	13.0	7.3	12.0	8.4	7.6	9.7
Whole Blood Glucose (mmol/l)	13.9	6.0	NA	NA	NA	9.0	10.6	14.7	12.0	11.5	6.9	9.8	7.6
Resting Calf Flow	2.4	2.0	3.5	2.2	2.5	2.0	5.3	NA	2.4	NA	2.5	2.5	2.5
Resting Foot Flow	0.5	0.09	2.0	0.2	0.09	2.0	2.5	0.5	1.5	0.5	0.09	2.0	1.0
Peak Post Stimulus Flow (Minus Resting Flow)	3.5	6.7	9.0	4.5	14.2	4.2	7.0	3.2	6.3	6.2	2.7	3.5	4.5
Time to Peak Flow (Seconds)	3.6	0.0	3.6	4.8	9.6	0.0	4.8	8.4	4.8	0.0	4.8	30.0	4.8
T <sub>50</sub> (Seconds)	18.0	36.0	45.6	24.2	19.8	9.0	7.8	12.0	24.0	18.0	20.4	122.0	28.8
O-30%	NA	500	50.0	185.0	NA	NA	37.0	NA	40.6	NA	500	38.0	53.7
Temperature °C	27.0	27.0	32.5	30.5	29.0	NA	30.5	NA	26.5	NA	26.5	31.0	25.4

TABLE 7 - 4

Median Peripheral Blood Flow Results of Normal Control  
Subjects and Diabetic Patients

	Normal Controls (N=16)	All Diabetic Patients (N=37)	Established Insulin Treated (N=15)	Established Tablet Treated (N=13)	Newly Diagnosed (N=9)
Resting Calf Flow	3.8	2.5	2.7	2.5	2.9
Resting Foot Flow	0.7	0.8	0.7	0.5	0.9
Time to Peak Flow	4.8	4.8	4.8	4.8	4.8
Peak Post Stimulus Flow (-Resting Foot Flow)	9.5	6.2**	6.5	4.5	6.2
T <sub>50</sub> (seconds)	20.4	21.6	21.6	24.0	20.4
0-30% (seconds)	67.2	72.6	75.0	52.0	77.0
Age (Years)	47.5	52.0	51.0	55.0	44.0
Haemoglobin A <sub>1</sub> C%		10.4	10.6	9.6	12.5
Duration (Years)		5.0	18.0	3.0	0.0
Blood Glucose at Time of Test (mmol/l)			15.0	10.2	6.1
Temperature (°C)	31.8		28.1	28.0	N/A

\*\* p 0.0001



TABLE 7 - 5

Correlations of Age and Foot Temperature  
with Peripheral Blood Flow in 16 Normal  
Control Subjects  
(P Values)

	Age	Temperature
Resting Calf Flow	0.4117	0.1118
Resting Foot Flow	0.3912	0.0007
Time to Peak Flow	0.8139	0.4028
Peak Post Stimulus Flow (Resting Flow)	0.4101	0.6893
T <sub>50</sub>	0.3519	0.0798
0-30%	0.0044 (+ ve)	0.3124

TABLE 7 - 6

Correlations of Age, Duration of Diabetes,  
Diabetic Control and Foot Temperature with  
Peripheral Blood Flow in 37 Diabetic Patients  
(P Values)

	Age	Duration	HbA <sub>1c</sub>	Glucose	Temperature
Resting Calf Flow	0.9163	0.5171	0.3650	0.6869	0.0670
Resting Foot Flow	0.5047	0.9008	0.1561	0.9815	0.0214
Time to Peak Flow	0.1102	0.1763	0.4943	0.7517	0.9260
Peak Post Stimulus Flow ( - Resting Flow)	0.8924	0.9252	0.9061	0.7732	0.8285
T <sub>50</sub>	0.1474	0.3032	0.5996	0.6137	0.1059
0-30%	0.9163	0.3957	0.1948	0.6238	0.0163

TABLE 7 - 7

Results of a Period of Improved Diabetic Control on  
Peripheral Blood Flow in 7 Newly Diagnosed Diabetic Patients

	S.B.	M.N.	L.C.	B.F.	Ri.K.*	C.R.*	A.B.*	Median
Haemoglobin A <sub>1</sub> C% 1) 2)	13.1	15.1	15.7	25.0	11.5	14.5	14.2	14.5
	11.2	9.8	12.5	17.8	9.9	8.5	12.5	11.2
Blood Glucose 1) (mmol/l) 2)	10.8	3.7	NA	NA	10.9	20.0	10.6	10.8
	6.0	4.0	NA	NA	7.9	6.9	2.6	6.0
Resting Foot 1) Flow 2)	0.2	0.09	2.6	2.9	0.4	0.8	0.5	0.5
	0.2	0.9	1.8	1.1	0.9	0.5	1.8	0.9
Peak Post 1) Stimulus 2) ( - Resting Flow)	4.2	12.8	12.0	9.0	5.6	4.5	4.0	5.6
	4.5	15.0	8.0	7.5	9.0	5.5	7.0	7.5
Time to Peak 1) Flow (seconds) 2)	0.0	0.0	0.0	0.0	4.8	3.6	21.0	0.0
	0.0	9.6	0.0	9.6	4.8	0.0	4.8	4.8
O-30% 1) 2)	131	80	31	21	185	41	65	65
	140	135	22	61	101	92	40	92
T <sub>50</sub> (seconds) 1) 2)	9.6	7.2	>100	>100	19.2	102	39.6	39
	16.2	73.2	4.6	57.6	20.4	14.4	100	20.4

\* Examined before commencing treatment with insulin on visit 1

TABLE 7 - 8

Median Peripheral Blood Flow Results of  
15 Established Insulin Requiring Diabetic Patients

	At Entry	After Run In	Treatment with BHI	Treatment with PPI
Haemoglobin A <sub>1</sub> C (%)	10.5	9.5	10.4	10.1
Blood Glucose (during test) (mmol/l)	15.0	14.5	12.6	13.7
Resting Calf Flow (ml/100 ml tissue/min)	2.7	3.1	3.5	3.4
Resting Foot Flow (ml/100 ml tissue/min)	0.7	1.6	1.5	0.7
Time to Peak Flow (Seconds)	3.6	5.1	3.6	3.6
Peak Post Stimulus Flow (-Resting Flow) (ml/100 ml tissue/min)	7.7	6.3	7.2	7.5
T <sub>50</sub> (Seconds)	21.6	34.2	62.4	25.2
Temperature of Foot (°C)	28.1	30.0	29.5	29.3

TABLE 7 - 9

Results of Laser Doppler Flow Meter and  
Plethysmograph Measurements of T.P.F.

		Laser Doppler	Plethys- mography
Subject		T.P.F. (Seconds)	T.P.F. (Seconds)
Normals	R.C.	15.0	6.0
	D.P.	20.4	4.2
	D.H.	19.2	6.0
	R.A.	21.5	0.0
	Median	19.8	5.1
Diabetic Patients	D.C.	42.0	3.6
	A.E.	55.2	4.8
	C.H.	55.2	3.6
	R.G.	27.0	4.8
	M.W.	178.8	3.6
	P.P.	25.2	16.8
	W.M.	84.0	3.6
	W.H.	66.0	6.0
	M.T.	75.6	4.8
	D.D.	46.8	3.6
	Median	55.2	4.2

**TABLE 7 - 10**

Correlations of Common Peroneal F Latency  
and Insulin Levels with Peripheral Blood Flow  
(P Values)

	Common Peroneal F Latency	Insulin Level
Resting Calf Flow	0.6273	0.5171
Resting Foot Flow	0.6042	0.9008
Time to Peak Flow	0.0484	0.1763
Peak Post Stimulus Flow ( - Resting Flow)	0.6076	0.9252
T <sub>50</sub>	0.2983	0.3032
0-30%	0.8831	0.3597
Temperature	0.3388	0.7490

## CHAPTER 8

### RESULTS OF PERIPHERAL NERVE FUNCTION STUDIES

#### 8-1 INTRODUCTION

Although treatment of established diabetic neuropathy is disappointing, many studies, in both experimental and clinical diabetes (Gregerson, 1967; Ward et al, 1971; Fraser et al, 1979; Graf et al, 1981; Hreidarsson, 1981; Sidenius & Jakobsen 1982) have established that some improvement in nerve conduction velocity is associated with improvement of blood glucose levels. In clinical practice, intensive insulin therapy, to achieve near normal blood glucose levels is usually the treatment of symptomatic neuropathy.

It has been suggested that reversible changes in autonomic nerve function may partly account for the changes in blood flow associated with uncontrolled diabetes mellitus. In the neuropathic foot, blood flow patterns are grossly altered, with evidence of arterio-venous shunting (Edmonds et al, 1981; Boulton et al, 1982). Autonomic nerve function is more difficult to assess quantitatively than peripheral nervous function, but changes in autonomic nerve function, which occur on improvement of diabetic control, have been shown to parallel those of peripheral nerve function (Fraser et al, 1979).

#### 8-2 AIMS OF THE STUDIES

The aims of the studies of peripheral nerve function were to establish, (in view of the clinical habit of prescribing intensive insulin therapy to achieve near normal blood glucose levels in the treatment of diabetic peripheral neuropathy):

- 1) whether treatment with the then newly available biosynthetic human insulin conferred any advantage as regards nerve function
- 2) whether there was any link in changes of blood flow patterns and nerve function tests.

#### 8-3 METHODS AND STATISTICAL ANALYSIS

Protocols used to recruit and manage patients during the studies the double-blind crossover study of PPI and BHI are given in chapter 2. The methods used in the assessment of electrophysiological function are given in chapter 3. Fourteen patients participated in the double-blind study of the effects of treatment with BHI and PPI on peripheral nerve function. Clinical and electrophysiological assessments of these patients were made on the following 4 occasions:

- (a) at entry to the study
- (b) after the 3 month run-in period on actrapid and monotard porcine insulin therapy and
- (c&d) after each 3 month randomized double blind treatment with (c) BHI or (d) PPI

In addition, four Type II diabetic patients treated with oral agents were studied once. This data was only used in the analysis of the relationship between peripheral nerve function and peripheral blood flow.

The results are expressed as means and analyzed by the Wilcoxon matched-pairs signed-rank test accepting a level of significant difference of 5%. The changes in peripheral nerve function tests occurring on treatment with BHI and PPI in the double-blind study were also analyzed using the Wilcoxon matched-pairs signed-rank test. Spearman's test of rank correlation was used to assess for correlations of peripheral blood flow measurements and peripheral nerve function tests.

#### 8-4 DETAILS OF DIABETIC PATIENTS STUDIED.

The patients in whom peripheral nerve function was studied were 14 of the 15 established diabetics with microvascular disease, who had agreed to participate in the double blind crossover study of biosynthetic human and purified porcine insulins. The missing patient, W.H. who participated in the studies of haemostatic function, failed to attend for electrophysiological examination. Details of these subjects are given in chapter 6, section 6-3, and in table 6-3. In addition, results of electrophysiological tests on 4 patients treated with oral hypoglycaemic agents (J.S., G.M., M.W., and M.D. details of whom are given in chapter 6, section 6-3, and in tables 5-2 and 6-4) were used in the analysis of the relationship of blood flow and peripheral nerve function. None of the 18 patients were on any agent known to interfere with peripheral nerve function and all reported light to moderate alcohol consumption (Drinking in England and Wales, 1980). All 18 subjects had retinopathy (confirmed by fluorescein angiography). Thirteen had clinical evidence of neuropathy (diminished soft touch, pinprick or vibration sense plus or minus ankle jerks) although only 2 patients, (G.B., R.K.) were symptomatic. All 18 patients had electrophysiological abnormalities consistent with neuropathy. Results of peripheral nerve function tests are given in Table 8-1.

#### 8-5 RESULTS OF THE DOUBLE-BLIND STUDY WITH BHI AND PPI

Results are given in Tables 8-2 (run-in period) and 8-3 (double-blind period). During the run-in period of improved blood glucose control preceding the double blind crossover study of BHI and PPI, the mean HbA<sub>1</sub>C fell significantly ( $p < 0.02$ ). The mean motor conduction velocities and F response latencies of the ulnar ( $p < 0.04$ ) and



common peroneal ( $p < 0.03$ ) nerves also improved during the run-in period but changes in sensory conduction velocities were less consistent: SCV of the ulnar nerve was unchanged although sural SCV did improve ( $p < 0.03$ ). During the double blind phase of the study blood glucose control was marginally worse on treatment with BHI than PPI ( $p = 0.049$ ). All nerve conduction velocities on treatment with PPI and BHI were the same, apart from the common peroneal MoCV, which was decreased on BHI treatment ( $p = 0.0157$ ). Throughout the study there was no clinical or symptomatic improvement in neuropathy, even in the run-in period.

#### 8-6 RELATIONSHIP OF PERIPHERAL NERVE FUNCTION TESTS AND PERIPHERAL BLOOD FLOW

Results of associations of peripheral nerve function tests and peripheral blood flow are given in Table 7-10 and discussed in Chapter 7.

#### 8-7 DISCUSSION

Neuropathy is a common cause of disability in diabetic patients. Many diabetic patients have electrophysiological changes and an estimated 25% will eventually develop symptomatic neuropathy (Clements, 1979). The available evidence in experimental diabetes suggests that if hyperglycaemia is completely reversed at an early stage, peripheral nerve function returns to normal pre-diabetic levels (Sidenius & Jakobsen 1982). In clinical diabetes mellitus this reversible "hyperglycaemic" component probably coexists with irreversible nerve damage. In this study, of long-standing diabetic patients, a small improvement in blood glucose control in the run-in period of the study resulted in significant improvement in nerve conduction velocities. However there was no improvement in neurological signs or symptoms. During the period of improved diabetic control, the F response latencies of the ulnar and peroneal nerves proved the most sensitive indicators of change with a 3.8% and 6.2 % improvement respectively compared with a 2.6% and 3.9% respective improvement in motor conduction velocities. Treatment with BHI was associated with a slight deterioration in blood glucose control and a tendency towards slowing of nerve conduction velocities, which was only significant with regard to common peroneal motor nerve conduction. If better diabetic control had been achieved on treatment with BHI, it is likely that nerve conduction velocities would also have improved. Treatment with BHI was not associated with any advantages with regard to peripheral nerve function.

**APPENDIX**

**TABLES TO CHAPTER 8**

TABLE 8 - 1

## Results of Nerve Conduction Tests in 18 Diabetic Patients

	INSULIN TREATED														ORAL THERAPY				
	P.F.	G.S.	R.K.	G.B.	G.T.	N.T.	W.M.	P.P.	A.E.	D.D.	M.Wi.	R.G.	C.H.	D.C.	J.S.	G.M.	M.We.	M.D.	
Ulnar Motor Conduction Velocity (Metre/Second)	54	53	54	50	50	48	45	52	52	50	45	50	44	52	52	52	52	52	
Ulnar Sensory Conduction Velocity (Metre/Second)	44	47	47	44	50	47	48	50	47	42	43	54	43	52	47	50	52	50	
Common Peroneal Motor Conduction Velocity (Metre/Second)	37	43	40	38	39	39	43	33	46	36	35	43	31	38	40	36	41	38	
Sural Sensory Conduction Velocity (Metre/Second)	40	38	42	N.R.	41	34	37	40	36	38	40	48	35	41	38	38	36	NR	
Ulnar F Response (Millisecond)	29.5	29.0	30.5	31.0	34.5	35.0	35.5	29.5	27.0	36.0	32.0	29.0	35.0	30.0	32.0	31.0	29.5	31.0	
Common Peroneal F Response (Millisecond)	54.0	52.5	61.0	58.0	55.0	61.0	66.0	70.0	51.0	65.5	59.5	57.0	NR	NR	58.0	63.0	49.0	NR	

NR - Not recordable

**Table 8 - 2**

Results of Nerve Conduction Tests at entry to the study and period of Improved Control in 14 Diabetic Patients  
(Mean  $\pm$  standard error)

	Pre Trial	Post Run In	p Value
HbA <sub>1c</sub> %	10.9 $\pm$ 0.48	10.1 $\pm$ 0.56	0.011
Ulnar Motor Conduction Velocity (metres/sec)	49.7 $\pm$ 0.92	51.0 $\pm$ 0.99	0.033
Ulnar F Latency (milliseconds)	31.9 $\pm$ 0.82	30.7 $\pm$ 0.72	0.028
Ulnar Sensory Conduction Velocity (metres/sec)	47.0 $\pm$ 1.04	48.1 $\pm$ 0.87	0.500
Common Peroneal Motor Conduction Velocity (metres/sec)	38.3 $\pm$ 1.15	39.8 $\pm$ 1.18	0.022
Common Peroneal F Latency (milliseconds)	58.3 $\pm$ 1.80	55.5 $\pm$ 1.76	0.005
Sural Sensory Nerve Conduction Velocity (metres/sec)	39.2 $\pm$ 1.00	37.5 $\pm$ 3.27	0.002

Table 8 - 3

Nerve conduction study results at the end of each three month period of treatment on PPI and BHI (means  $\pm$  standard error of the mean) and mean changes in nerve conduction studies on treatment with PPI -  $\Delta$ PPI and on treatment with BHI -  $\Delta$ BHI

	PPI	BHI	p	$\Delta$ PPI	$\Delta$ BHI	p
HbA <sub>1c</sub> %	9.9 $\pm$ 0.52	10.6 $\pm$ 0.43	0.049	+0.217	-0.542	0.182
Ulnar motor conduction Velocity (Metres/sec.)	51.1 $\pm$ 0.98	50.21 $\pm$ 0.88	0.209	-1.077	+0.385	0.374
Ulnar F latency (milliseconds)	30.9 $\pm$ 0.68	30.8 $\pm$ 0.73	0.665	-0.307	0	0.600
Ulnar sensory conduction Velocity (metres/sec.)	47.6 $\pm$ 0.70	47.9 $\pm$ 0.92	0.9	+0.385	-0.154	0.552
Common peroneal motor conduction velocity (metres/sec.)	40.9 $\pm$ 0.90	39.1 $\pm$ 1.02	0.016	-0.923	+1.846	0.017
Common peroneal F latency (milliseconds)	56.2 $\pm$ 1.23	58.0 $\pm$ 1.69	0.286	-0.654	-1.73	0.900
Sural sensory conduction Velocities (metres/sec.)	40.6 $\pm$ 1.07	39.2 $\pm$ 1.36	0.182	+3.0	+1.53	0.782

## CHAPTER 9

### ASSESSMENT OF RETINAL STATE

#### 9-1 INTRODUCTION

All established diabetic patients participating in the double-blind crossover study of treatment with biosynthetic human and purified porcine insulins on haemostatic function, blood rheology and peripheral blood flow were selected on the basis of the presence of microangiopathy when screened in the diabetic eye clinic by an experienced ophthalmologist. These patients subsequently had plain fundal photographs and fluorescein angiography of the retinal vessels at entry to and at the end of the studies to assess progression of retinopathy. The fundal photographs and retinal fluorescein angiograms were examined and reported by a second ophthalmologist. Full details of the photography and fluorescein angiography are given in chapter 3.

#### 9-2 RESULTS

In all, only 9 out of 15 patients who participated in the double-blind crossover study had fully assessable retinal angiograms. Plain fundal photographs and fluorescein angiograms were not available on 2 patients at entry to the study because of staff shortages. Both these patients (C.H. and R.G.) were considered to have simple background retinopathy on screening with an ophthalmoscope by the ophthalmologist. In addition, one patient (W.H.) refused fluorescein retinal angiography and only had one set of plain fundal photographs taken because he found the procedure uncomfortable. In 2 patients (M.W. and M.T.), the fluorescein angiograms were of poor quality at both visits and in one other (W.M.), the angiograms were of poor quality at the second visit. Because of these technical problems, elaborate quantitative methods of assessment of the fundal photographs and retinal angiograms (as recommended by Baudoin et al, 1983) were not considered worthwhile.

Of the 15 patients participating in the double-blind crossover study of biosynthetic human and purified porcine insulins, 4 had required photocoagulation treatment before entry into the study.

D.C. to both eyes for proliferative retinopathy, first treatment 3 years before entry to the study. On screening at entry to the study new vessels were noted and she was placed on the waiting list for further photocoagulation treatment.

P.P. to the right eye for treatment of maculopathy one month before entry to the study (left eye background changes only).

W.H. to both eyes for proliferative retinopathy, first treatment 4 years before entry to the study, last treatment 2 years before study.

D.D. had received multiple treatments for proliferative retinopathy to both eyes, first treatment 4 years before study, last treatment within a year before study.

The remaining patients were all considered to have simple background retinopathy at entry to the study with the exception of R.K. who had numerous microaneurysms, dot haemorrhages, hard exudates and areas of capillary non-perfusion in both eyes.

During the course of the study, 3 patients required laser photocoagulation treatment:

R.K. the changes in the eyes became more florid during the 3 month run-in period of the study and he required laser photocoagulation treatment to both eyes.

G.B. developed maculopathy in the left eye and received photocoagulation treatment 6 months after entry into the study.

D.C. had laser coagulation of new vessels in the right eye 2 months after entering the study and subsequently suffered a vitreous haemorrhage in the same eye.

There was no deterioration or improvement in any of the eyes of the remaining patients who had simple background retinopathy at the time of entry to the study. Neither W.H. or D.D., who both had received laser photocoagulation treatments remained stable throughout the study period. Details of the assessment of individual eyes are given in Table 9-1.

### 9-3 DISCUSSION

Although initial reports suggested that diabetic retinopathy could be reversed by improvement of diabetic control (Irsigler et al, 1979), accumulating recent reports provide evidence of deterioration of existing retinopathy particularly in patients in whom rapid return to near normoglycaemia is achieved by treatment with continuous infusion or multiple subcutaneous injection of insulin (Hooymans et al, 1982; Lauritzen et al, 1983; Kroc Collaborative Study Group, 1984; Dahl-Jorgensen et al, 1985). Such regimens would appear to result in improved retinal function but morphological deterioration (Lauritzen et al, 1983). In this study, gentle improvement of diabetic control was achieved during the run-in period of the study on twice daily subcutaneous soluble and intermediate acting insulin regimens. The initial improvement of diabetic control in the first three months of the study was however not maintained. Of the 15 patients studied, deterioration of diabetic retinopathy occurred in 3 individuals of whom 2, R.K. and G.B., showed deterioration of retinal morphology and in the third bleeding occurred from new vessels. The small number of patients involved and lack

of a control group means that no significant conclusions can be drawn as to the effects of participating in the study on progression of diabetic retinopathy.

Whilst it is generally believed that degree and duration of exposure to hyperglycaemia increases the risk of developing retinopathy, the paradoxical risks of improving diabetic control are becoming apparent. Amongst the reasons put forward to explain deterioration of established background retinopathy with improved diabetic control are first, that after a certain critical level of damage has occurred diabetic retinopathy is irreversible; secondly that the damaged retinal circulation has become dependent on the high flow rates associated with hyperglycaemia to maintain flow through sclerosed vessels and that reduction of hyperglycaemia and blood flow results in retinal ischaemia and thirdly that the regimens used to obtain good diabetic control may themselves be harmful, for instance, by increasing episodes of hypoglycaemia (Dahl-Jorgensen, 1985). Thus in the early stages of diabetes, strict control of blood glucose levels would appear to protect from the development of retinopathy but once damage has occurred the appropriateness and benefits of attempting to achieve normoglycaemia require cautious assessment. Despite these reservations, in the absence of any other pharmacological option, normoglycaemia remains the treatment of choice for preventing new diabetic damage. Identification of areas of ischaemic retina and early treatment may help prevent paradoxical deterioration of retinopathy on improving diabetic control.



**APPENDIX****TABLES TO CHAPTER 9**

Table 9-1

## The Assessment of Retinal State by Retinal Photography and Fluorescein Angiography

SUBJECT	G S		G T		R K		G B		P F		D C		A E		C H		R G		M W		P P		W H		M T		W H		D D	
	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L
Microaneurysms	+	+	+	+	++	++	+	+	+	+	+	+	+	+	NA	NA	NA	NA	+	+	+	+	+	+	+	+	+	+	+	+
	++	++	+	+	-	-	-	-	+	+	NA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Haemorrhages	+	+	+	+	+	+	+	+	+	+	+	+	+	+	NA	NA	NA	NA	+	+	-	-	-	-	-	-	-	-	-	-
	+	+	-	-	-	-	-	-	+	+	NA	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Hard Exudates	-	-	-	-	+	+	-	-	+	+	+	+	+	+	NA	NA	NA	NA	+	+	+	+	+	+	+	+	+	+	+	+
	-	-	-	-	-	-	-	-	-	-	NA	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Capillary Non-Perfusion	+	+	-	-	+	+	-	-	NA	NA	+	+	-	-	NA	NA	NA	NA	+	+	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cotton Wool Spots	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NA	NA	NA	NA	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
New Vessels	-	-	-	-	-	-	-	-	-	-	+	+	-	-	NA	NA	NA	NA	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Intra Retinal New Vessels	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NA	NA	NA	NA	+	+	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Retinal New Vessels	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NA	NA	NA	NA	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Vitreous New Vessels	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NA	NA	NA	NA	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Laser Photocoagulation Scars	-	-	-	-	+	+	-	-	-	-	-	-	-	-	NA	NA	NA	NA	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	+++	+++	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pre-Proliferative (P.P.) Background (B) Proliferative (P) Treated (T) Vitreous Haemorrhage (V.H.) Not Assessable (N.A.)	B	B	B	B	P.P.	P.P.	B	B	B	B	P	P	B	B	-	-	-	-	B	B	B	B	B	B	B	B	B	B	B	B
	B	B	B	B	T	T	B	B	B	B	NA	NA	T	T	B	B	B	B	B	B	T	T	B	B	B	B	B	B	B	B

## CHAPTER 10

### CONCLUSIONS AND IMPLICATIONS FOR FUTURE RESEARCH

Vessel changes in both large and small vessels are the most important longterm complications of diabetes mellitus. Whilst it is accepted that severity and length of duration of exposure to hyperglycaemia are the most important predictors of the development of diabetic vascular complications, the pathogenetic mechanisms remain unclear and consequently no specific preventative measures have been developed.

Changes in 1) blood rheology, 2) haemostatic function and 3) the regulation of blood flow in diabetes mellitus have all been suggested as contributing to the development of vascular damage. In this thesis the relationship of these factors to 1) the presence of diabetes mellitus, 2) the degree of hyperglycaemia, 3) improving diabetic control and 4) treatment with Biosynthetic Human Insulin have been studied.

The studies have shown that many factors, such as whole blood viscosity and levels of various haemostatic factors are positively related to the severity of hyperglycaemia and that a modest improvement in diabetic control, as can be achieved by closer outpatient supervision, may result in small changes of some factors. Treatment with Biosynthetic Human Insulin does not confer any advantage as regards blood rheology, haemostatic function, peripheral blood flow or peripheral nerve function.

The development of diabetic vessel disease is undoubtedly multifactorial. The concept of the diabetic whose blood vessels are stressed by the passage of more rigid erythrocytes, whose hyperviscous blood impairs flow in the microcirculation, whose blood clots more readily but whose fibrin clots are removed less efficiently because of an impaired fibrinolytic capacity, might, in itself, appear to provide an adequate explanation for the predisposition of diabetics towards vascular disease. Is it possible that these changes alone are the cause of blood flow disturbances and eventual vascular damage?

The contention that abnormal blood rheology contributes to impaired perfusion of the microcirculation found little support in the studies presented here, as the filtration method used to determine erythrocyte deformability was not specific for erythrocytes. Previous studies, which have used filtration methods to assess erythrocyte deformability, and on which the evidence for reduced erythrocyte deformability in diabetes mellitus are largely based, are probably invalid. Only the development and application of new methods of measuring erythrocyte deformability will solve this issue. In addition, the cell of interest may prove to be the leucocyte, rather than the erythrocyte.

The overall rheological properties of blood were assessed by measuring whole blood viscosity. A mild elevation of whole blood viscosity was recorded in diabetic patients, but no obvious association of whole blood viscosity and blood flow was found, although there was an association in normal controls between whole blood viscosity and resting forefoot flow. The plethysmographic method used to assess peripheral blood flow was not as sensitive as the Laser Doppler Flowmeter to changes in microcirculatory blood flow. It might be interesting to study the relationship between skin microcirculation (with the Laser Doppler Flowmeter) and whole blood viscosity in normals, diabetics and non-diabetic patients with hyperviscosity syndromes. Recent theoretical analysis (Rampling & Challoner, 1983) suggests that raised fibrinogen (which largely accounts for the increased viscosity of diabetes not accountable for by haematocrit) is not an important determinant of flow under normal physiological conditions. Perhaps in diseased vessels, where flow rates are low, this increase in whole blood viscosity may have more noticeable effects, and may further hinder flow. It seems unlikely that the small increase in whole blood viscosity, described in diabetes, plays more than an aggravating role in the development of microvascular damage. In one of the few available investigations of in-vivo rheology, Branemark et al (1971) did not find any impairment of erythrocyte deformability and blood flow, except in diabetic ketoacidosis, where erythrocyte entry into capillaries was sluggish. Further in-vivo studies of blood flow in relation to alterations of blood rheology are required.

No indisputable evidence of a hypercoagulable state in diabetes mellitus was found, although elevation of certain haemostatic factors was noted. Whether these changes contribute towards vessel damage in diabetic patients is still a subject of conjecture. It seems unlikely that the coagulation changes described in this thesis, namely of raised levels of fibrinogen and factor VIII, von Willebrand factor, could cause vessel thrombosis under normal conditions. But on interaction with damaged endothelium, these changes may favour clot formation. Impaired fibrin clearing mechanisms might tip the balance towards vessel occlusion. Fibrin deposition has been shown to stimulate smooth muscle proliferation, and is a possible factor in the development of atheromatous lesions (Kadish et al, 1979; Ishida & Tamaka, 1982). It has also been noted, that large amounts of fibrin are found in the atheromatous lesions of arteries (Haust et al, 1965) and diabetic patients are more prone to the development of atheroma which is generally of a more extensive nature than in non-diabetic patients (Kramer & Perilstein, 1958). In small vessels, histological studies have revealed the presence of intravascular coagulation, in both neural (Timperley et al, 1976) and in cerebral small vessels (as reviewed by West, 1982). Fresh fibrin deposits are seen in the vasa nervorum following diabetic ketosis, and whilst this is not necessarily the cause of nerve damage, obstructed vessels are also found in established diabetic neuropathy. It might prove interesting to study the relationships between haemostatic

factors, vessel wall state and clot weight, size and clearance following artificial arterial damage in diabetic and non-diabetic animals.

As it seems unlikely that changes in blood rheology and haemostatic factors described initiate the development of diabetic microangiopathy, attention must turn to the blood vessels themselves. Is it possible that there is a primary, initially reversible, degeneration of small blood vessels in diabetes, and that the changes in blood rheology and haemostatic factors described are secondary? The presence of raised levels of von Willebrand Factor may be a marker for the presence of vascular damage - its main site of storage is the endothelium (as reviewed by Porta et al, 1987) and fibrinogen (which largely accounts for the raised blood viscosity found in diabetes mellitus) is an acute phase reactant protein. Thus changes in blood rheology and haemostatic factors, described in this thesis, could be entirely explained as markers for the presence of vascular disease. The presence of deteriorating retinopathy was an independent predictor for higher levels of both factors and other independent predictors such as high HbA<sub>1c</sub>, high blood pressure and long duration of diabetes mellitus are also known risk factors for the development of vascular disease. Abnormalities of vasodilation were found on both plethysmographic and Laser Doppler Flowmeter studies of foot blood flow and may represent a defect of the microvessels, although alterations in other factors influencing blood flow such as autonomic nerve function, and hormonal and metabolic factors may have affected regulation of blood flow. Further studies to elucidate the nature of the flow defect in diabetes mellitus and to establish the importance of the interaction in-vivo of alterations in blood rheology and coagulation factors are required.

The presence of an abnormal test or raised level of a particular factor is not evidence of a role in the development of late diabetic complications. It has been argued that the correlation of a factor with the presence of hyperglycaemia is more in favour of the factor being a simple marker of hyperglycaemia rather than signifying any importance in the genesis of vessel damage. Greater confidence could be attributed to a causative relationship if the abnormality of the suspect factor were more marked at a given level of diabetic control, in those patients who subsequently develop vessel complications. In this respect, very large, longterm prospective studies of suspected pathogenic factors and the development of diabetic vascular damage may be considered a better guide of pathogenic factors, than the relatively small, shortterm studies presented in this thesis. However causative relationships are best established by longterm intervention studies using agents which selectively affect factors under study. Examples of such specific intervention studies include the treatment of blood pressure and the progression of diabetic nephropathy (Parving et al, 1983 [a]); this study supports the hypothesis, that there is a failure of autoregulation of capillary perfusion (under changing pressure heads) and subsequent capillary hypertension and damage in diabetes, since lowering

blood pressure slows the progression of nephropathy. Another intervention study is the successful treatment of diabetic neuropathy with aldolase reductase inhibitors (Judzewitsch et al, 1983) a finding which suggest a role for abnormal metabolites in the causation of neural damage.

Unfortunately, there are few acceptable agents with which to perform such studies on the effects of altering levels of blood haemostatic factors, blood viscosity or peripheral blood flow in diabetes mellitus.

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Ritchie, D.M. (1985) Filtration of Dilute Erythrocyte Suspension as a Measure of Erythrocyte Deformability and its Relation to Blood Glucose Control in Diabetes Mellitus. *Clinical Hemorheology*, 5, 257-268.